Role of protein charge in reversed micellar extraction

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Role of protein charge in reversed micellar extraction

Forney, Craig Everett, Ph.D.
Iowa State University, 1994
Role of protein charge in reversed micellar extraction

by

Craig Everett Forney

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
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Iowa State University
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1994
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INTRODUCTION

The advent of recombinant DNA technology has given scientists the ability to manipulate the production of proteins from microorganisms. This has resulted in the production of a wide variety of products, ranging from medications, such as tissue plasminogen activator, to enzymes used in food production, such as rennin. It has also given scientists the ability to modify protein properties such as a protein's pH or thermal stability or its tolerance to organic solvents. Despite the advances that these techniques have given in these applications, in only a few cases have recombinant DNA techniques been used to address a significant problem in bringing a biological product to market: the separation and purification of the target protein from the fermentation broth to homogeneity. In this work, we have used genetic modifications in the form of charged fusion proteins to explore the effects such modifications would have on one purification method, reversed micellar extraction.

A series of mutations were made to the enzyme glucoamylase from Aspergillus awamori consisting of negatively charged peptides fused to both the amino and carboxyl termini of the protein. The fusions to the amino terminus were of the form: Asp-Tyr-(Asp),Lys-Ala-Arg, with n = 4 and 9 for GA'ND5 and GA'ND10. The carboxyl terminus fusions had the following sequence: Met-Ala-(Asp),Tyr with n = 0, 5 and 10 for GA'CD0, GA'CD5, and GA'CD10, respectively. Both of these series of mutants were based on a truncated form of the wild-type enzyme consisting of 482 amino acids (Forney and Glatz, 1994).

The objective of this work was to study the effects of charged fusion proteins on extraction behavior. Specific questions to be answered were:

1. Does the presence of the additional charged residues increase the amount of protein extracted from an aqueous phase relative to the control version?
2. Can using a charged fusion increase the selectivity of the process towards the target
protein?
3. Does the presence of the charged fusions alter the stability of the protein in the reversed micelle?
4. Could reversed micellar extraction be used to recover the fusions directly from clarified cell broth?

Dissertation Organization

The dissertation contains three main sections. The first section provides a literature review to introduce the reader to protein separation techniques in general, reversed micellar extraction in particular, and the use of charged fusions to enhance selectivity of separation processes.

The next section consists of experimental work presented as journal manuscripts. The first paper concerns the use of charged fusion proteins in reversed micellar extraction, emphasizing one particular surfactant system, trioctyl methyl ammonium chloride. The second paper focuses on comparisons between multiple surfactant systems, with emphasis on improving the recovery of glucoamylase from the reversed micelles and an attempt to unify the results from the two surfactants used. Recovery of glucoamylase from clarified cell broth is also covered here. The third paper is on the development of a technique used to purify glucoamylase fusion proteins from clarified, concentrated, and diafiltered cell broth. The work was performed under my supervision by two undergraduates working in our laboratories, Deborah Stafslien and Jeff Hunter, and the paper was primarily written by me with their input.

The main body closes with the overall conclusions from the project, followed by an appendix. The appendix gives the basics of protein charge estimation and the estimated net charge of the control version of glucoamylase as well as some of the mutants used in this work as a function of pH.
Protein Separation

The separation processes surrounding the purification of proteins for pharmaceutical, food, or industrial use are complex. The complexity lies in the physical properties of the whole fermentation broth: it is very dilute, containing cells and cell debris, metabolites, salts, nucleic acids, many proteins (including the desired product), and other materials.

An overall separation process must, therefore, consist of a series of steps, each designed to remove one/several of the components mentioned above in a systematic way. In a general process, there are four basic steps, each of which may include several different methods. The four steps are removal of insolubles, isolation of products, purification of the products, and polishing (Belter et al., 1988).

The primary purpose of the first step is to remove the insolubles from the fermentation broth. The purpose for this is twofold. For extracellular proteins (those which are excreted), the effect is to remove substances that would complicate further separation steps; for intracellular proteins (those not excreted), this step serves to concentrate the product into a much smaller volume so that in the subsequent steps the volume handled and the required equipment can be much smaller. Typical techniques for insolubles removal are filtration and centrifugation.

The next step in a separation process is to isolate the products from other broth components that have properties widely different from the product. Lipids from cell membranes and nucleic acids have properties that are much different from proteins, and these differences can be used to remove them from the solution. One example of such a step would be using solvents to extract the lipids from a broth.

The next step is to purify the product from solutes with similar properties. The similar properties might be solubility, density, charge at a given pH, etc. There are many
techniques available for this step, including precipitation, chromatography, electrophoresis, and extraction. These techniques will be discussed in more detail later. The final general step in a separation process is to polish the product, to put it in its final form. Typical steps for this are crystallization and drying, with end use determining polishing technique.

In this research we have been most interested in the second and third steps, isolation and purification of the proteins. Specifically, we wanted to see if we could develop processes that both purify and concentrate our product. At the outset, this does not appear to be too difficult a task, but when we examine the protein itself more closely, we see that proteins are quite labile. Protein products must be in their native, active conformation to be useful (except in the case of a feed supplement, where the mere presence of the amino acids that make up the protein is important). Proteins can be denatured by temperature and pH extremes, solvents (particularly organic ones), foaming, etc., and so we must be careful in choosing our purification steps. Distillation would be of little use in protein separation, as proteins are not volatile. Conventional filtration is of little use, as it could not possibly distinguish between proteins. Liquid-liquid extraction would appear to be a possibility as there are differences in hydrophobicity between proteins, but the organic solvents used might denature the proteins by penetrating into their hydrophobic core and upsetting the secondary, tertiary, and quaternary structure. Obviously, we cannot call upon the bulk of traditional chemical engineering separation methods when dealing with proteins.

Because of the protein's fragility, separation methods particularly appropriate for bioseparations have been developed. Above we mentioned precipitation, chromatography, electrophoresis, and (aqueous two-phase) extraction. Each of these methods has its own strengths and weaknesses. Recall that our starting broth is very dilute and contains many components with similar properties. Because of this, our ideal separation method would both concentrate our protein and be selective for it. The sad truth is that except for affinity methods
and a few rare other cases, these objectives are mutually exclusive.

Precipitation, be it through salt, acid, organic solvent, or polyelectrolyte, generally concentrates but is not particularly selective. Elution chromatography has the potential for great selectivity, be it through ion exchange, reverse phase, gel permeation or affinity chromatography, but often it dilutes the product during column elution. Ultrafiltration and the other membrane processes can concentrate proteins but most often are selective for a particular protein only through a size-exclusion effect. Electrophoresis is, for the most part, a small, laboratory scale technique that is effective but difficult to scale up. Aqueous two-phase extraction has much promise as a separation step, but much work needs to be done to develop the process.

Reversed Micellar Extraction

One method that could potentially be both selective towards and also concentrate proteins is reversed micellar extraction of proteins. Reversed micelles are aggregates of surfactant (surface active agent) molecules that surround water pools in organic solvents. Proteins, along with other molecules, may be solubilized in these water pools, primarily through electrostatic interactions between the head groups of the surfactant molecules and charged amino acid residues on proteins.

Mechanism of Extraction

Extraction of proteins from an aqueous phase into a reversed micellar organic phase can be seen as the combination of several steps. The first of these is the diffusion of the protein from the bulk aqueous phase to the aqueous/organic interface. This step has recently been found to be rate limiting for forward transfer (Dungan et al., 1991). Consequently, resistance to mass transfer can be minimized by making that diffusional distance as small as possible by providing good mixing to the system, thus reducing the droplet size. The second step in this process is the incorporation of the protein into a reversed micelle. This is assumed to take
place via a cooperative mechanism where the interface between the two phases deforms about the protein as it approaches the interface, shown schematically in Figure 1 (Dungan et al., 1991). In this picture, solubilization is driven by electrostatic interaction between the protein and the charged headgroups of the surfactant. This interparticle force is composed of attractive interactions between oppositely charged surfaces and osmotic repulsion from the concentration of counterions (required for electroneutrality) that grows with decreasing separation distance between the two surfaces. This is shown schematically in Figure 2.

On the other hand, it has been shown by both Dekker et al. (1990) and Dungan et al. (1991) that interfacial processes dominate the resistance to mass transfer for back extraction, not diffusional resistance. Dungan et al. (1991) break down the back transfer into two steps, that of coalescence of the protein-laden reversed micelle with the interface and disengagement of the protein from the interface. This mechanism is shown in Figure 3. It is expected that this second step, disengagement of the protein from the interface, would be subject to electrostatic interactions similar to that for forward transfer, with environmental conditions determining if disengagement or resolubilization is the predominant step. Dungan et al. (1991) estimate that the rate-determining step for back transfer is the coalescence step.

Factors Affecting Solubilization

Göklen and Hatton (1986) were able to separate a mixture of three proteins into separate, pure components using reversed micellar extraction. Obviously, in order to do this, there must be a way to selectively partition biomolecules. There are a number of parameters that may be used to vary the selectivity of the process, mostly related to electrostatic effects, but also due to the solvent. The four main things that can be used to effect partitioning are the choice and concentration of the surfactant and cosurfactant (if one is used), the charge on the protein (as determined by the aqueous phase pH), and the ionic strength and ion type. Each of the parameters will be discussed in more detail below. Additional factors would include the
Figure 1. Cooperative mechanism proposed for protein solubilization by a reversed micellar droplet (after Dungan et al., 1991).
Figure 2. Schematic representation of interparticle force profile as a function of flat plate separation distance (after Dungan et al., 1991)
Figure 3. Processes involved during protein desolubilization. (a) Diffusion of filled micelle to interfacial region, (b) coalescence of filled and empty micelles (with mixing of contents), (c) coalescence of empty micelles, (d) coalescence of empty micelle at interface, (e) coalescence of filled micelle at interface, and (f) disengagement of protein from interface (after Dungan et al., 1991).
type of organic solvent, the temperature of the solutions, and the particular properties of the proteins, but these will not be covered specifically here.

**Surfactant** Three basic types of surfactants have been used for the formation of reverse micelles. To selectively partition proteins, the first step is to choose a surfactant that has a charge opposite to that of the protein of interest. For a protein with a low pI (and correspondingly a negative charge at neutral pH's), the best choice would be to use a cationic surfactant. The opposite is true for a protein with a high pI. If the protein is stable both below and above its pI, one could choose the surfactant based upon the characteristics of the remaining solutes.

The choice of surfactant also affects things other than the charge of the inner layer of the micelle. The size of the reverse micelle is greatly affected by the choice of surfactant. Anionic surfactants generally form larger micelles than do cationic ones. This property is important for solubilizing large proteins as surface tension considerations create a size exclusion effect. Also affected by the type of surfactant is the energy required to enlarge the reversed micelles, a process that apparently occurs when a protein is solubilized. In addition, the charge density on the inner surface of the micelle will be dependent upon the type of surfactant (Dekker et al., 1989).

Generally, cationic surfactants require a cosurfactant to form larger micelles, with those cosurfactants typically being long chain alcohols. Anionic surfactants do not require a cosurfactant to form large aggregates (Castro and Cabral, 1988). This is described by Luisi and Magid (1986) using a surfactant packing parameter, \( \frac{v}{\alpha l} \), where \( v \) is the volume of the hydrocarbon chain of the surfactant, \( \alpha \) is the head group area, and \( l \) is the optimal length of the hydrocarbon chain (close to that of the fully extended chain). When this packing parameter is larger than one, the solution stabilizes the formation of reverse micelles. This occurs naturally for anionic surfactants such as AOT, but in the case of cationic surfactants, a cosurfactant is
needed to increase $v$ with only a small increase in $a$, resulting in the parameter becoming greater than one.

The use of two surfactants at once has been tried by Dekker et al. (1987) to test the effect of nonionic surfactants on the extraction efficiency and partitioning behavior of a protein. They found that the partitioning behavior was a strong function of the ratio of the nonionic surfactant to the cationic surfactant and that the net effect was that the phase transfer occurred over a wider range of pH's and the partition coefficient was much higher. The reasons they give for this are that the charge density of the micellar interface was affected and the size and flexibility of the reversed micelles was changed by the addition of the single-tailed nonionic surfactant (Dekker et al., 1989).

Another consideration is the concentration of the surfactant in the organic solution. Fletcher and Parrott (1988) found that the partitioning of chymotrypsin increased with increasing AOT concentration. In fact, a model for the partitioning of proteins into AOT has been made that uses the pH of the aqueous solution and the surfactant concentration as its variables (Woll and Hatton, 1989). This effect of increasing partitioning with increasing surfactant concentration is most likely due to a larger number of reversed micelles and not to larger reversed micelles (Dekker et al., 1989; Woll and Hatton, 1989).

Fletcher and Parrott also found that various proteins partition differently in response to changing surfactant concentration. The partitioning of chymotrypsin increased with increasing surfactant concentration, but at no point did this affect the stability of the protein remaining in the aqueous phase. However, with lysozyme they found that below a certain surfactant concentration (that required to give monolayer coverage to all of the protein in the aqueous solution) the protein that was not taken into the reverse micelles precipitated at the aqueous/organic interface (Fletcher and Parrott, 1988).
Protein Charge

As initially mentioned in regard to surfactant choice, appreciable solubilization only occurs when the surfactant and protein have opposite charges. The most direct way to affect the charge on the protein is to change the pH of the solution, and as such, this method has been used often to check the “charge” dependence of the partition coefficient (Meier et al., 1984; van't Riet and Dekker, 1984; Göken and Hatton, 1986; Fletcher and Parrott, 1988). Typical behavior is shown in Figure 4, where positively-charged proteins partition into negatively-charged AOT reversed micelles at pH values below the pI of the protein.

Using data from 19 proteins, Wolbert et al. (1989) found a correlation between the molecular weight of the protein and the difference between the optimal extraction pH of the aqueous solution and the pI of the protein. This is shown in Figure 5. The correlation for TOMAC micelles is

\[
(pH_{\text{opt}} - pI) = -0.97 + (1.1 \times 10^{-4}) \times MW
\]

and the corresponding correlation for AOT micelles is

\[
(pH_{\text{opt}} - pI) = -1.07 + (1.2 \times 10^{-4}) \times MW
\]

Wolbert et al. conclude that, for both cationic and anionic surfactants, “the size of the protein determines the charge density required for its transfer and thereby the pH of the uptake.” They explain this with the rationale that for proteins that are larger than the size of the unfilled micelle, there must be considerable rearrangement of unfilled micelles to accommodate the protein. This rearrangement will be energetically unfavorable, and so to compensate for this, there would need to be more extensive electrostatic interactions between the protein and micelle inner layer. This increase in electrostatic interactions shows up in a greater difference between the pH and the pI of the protein (Dekker et al., 1989).

Wolbert et al. (1989) found that there is a relationship between the asymmetry of the protein's charge distribution and the partitioning behavior. Specifically, they found that
Figure 4. Typical pH dependent behavior for extraction of proteins into an Aerosol-OT (AOT) reversed micellar phase. Solubilization of (■) cytochrome c (pI 10.6), (○) ribonuclease a (pI 7.8), and (▲) lysozyme (pI 11.1). Data taken from Göklen and Hatton (1987).
Figure 5. Relationship between protein molecular weight and the difference between the pI and the pH of optimal extraction in TOMAC reversed micelles. Data taken from Wolbert et al. (1989).
the more asymmetric the charge distribution was, the better it partitioned. This effect could
make charged fusions advantageous since they should provide high charge asymmetry.

Ionic Strength In accord with the electrostatic role already observed, one
would expect that there would be an effect due to the ionic strength of the solution, i.e. through
Debye screening, or through direct ion-pairing as suggested by Hatton (1989). Indeed, one
does find that the ionic strength of the aqueous phase can play a dominant role in the uptake of
proteins. Specifically, it has been found that ionic strength has two main effects: it decreases
the electrostatic interaction between the surfactant head groups and the protein and it also
reduces the electrostatic repulsion between surfactant head groups (Dekker et al., 1989),
resulting in smaller reversed micelles. The size reduction has been shown to be true by Sheu et
al. (1986), who used small-angle neutron scattering (SANS) to determine the size of filled
and unfilled AOT reverse micelles. The net result of the first effect is that the electrostatic
potential required for partitioning will increase with increasing ionic strength. Figure 6 shows
that increasing the salt concentration decreases the amount of protein solubilized at a given pH.

This effect of decreasing the interaction can be used to one's advantage when
attempting to back extract the proteins out of the micellar solution. The most common
procedure for removing proteins from reverse micelles is to contact the protein-laden organic
phase with an aqueous phase that has a pH close to the pi of the protein and increased ionic
strength, around 0.5M.

Ion Type Leser et al. (1986) observed that the partition coefficient for a protein
was affected by both the ionic strength and the ion types of the aqueous phase. For the system
AOT/isoctane, protein partitioning to the organic phase was strongest when calcium chlor­
ride was used in the aqueous phase, in comparison with either potassium or magnesium chlor­
ride. They did not attribute this effect to any particular ion characteristic, and so it may well
be due to specific interactions between the ions and the protein and/or surfactant head groups.
Figure 6. Effect of ionic strength on the partitioning of proteins in reversed micelles. Proteins are (■) cytochrome c, (○) ribonuclease a, and (▲) lysozyme. Data taken from Göklen and Hatton (1987).
Leodidis and Hatton (1989a, 1989b) have undertaken a more systematic approach in their study of the effect of salt type on protein partitioning in AOT reverse micelles. This study was broken down into two experimental sections plus an attempt to model this process. First they measured the partitioning of water and cations into the water pools to determine the effect of the ions on the micelle size in the absence of protein. Secondly, they measured the partitioning of proteins from an aqueous phase to the organic phase as a function of cation type. Finally, they attempted to model the concentration of ions in water pools as a function of ion type using a phenomenological approach.

They found that the minimum cation concentration necessary for micelle stability differed between cations. In addition, the absolute amount of water transferred depended on the cation. These differences were often significant, even when the ions had a similar valence and nearly equal hydrated size. Leodidis and Hatton also observed that among anions with similar valence, the anion type played very little role in the water uptake, but divalent anions led to slightly larger micelles than monovalent anions.

They also found that smaller cations were preferentially taken up over larger cations. As might be expected, divalent cations were taken up preferentially over monovalent cations, presumably on the basis of higher electrostatic interactions. Between divalent cations, there was a significant difference in behavior despite the fact that the hydrated sizes are nearly identical. Leodidis and Hatton attribute this effect to differences in hydration free energies (1989a). All in all, their experiments led them to the conclusion that distribution of the cation depends only on specific ion parameters such as charge, hydrated size, and polarizability, and not on other system variables, like surfactant concentration, phase volume ratio, etc.

In the second part of their study (1989b), Leodidis and Hatton observed the partitioning of proteins as a function of cation type. A plot of this is shown in Figure 7. What is significant although not unexpected about this plot is that the amount of protein transferred
Figure 7. Effect of salt concentration and ion type on α-chymotrypsin partitioning into AOT reversed micelles at pH 8.2. Ordinate is salt concentration of (■)NaCl, (●)KCl, (▲)CaCl₂, or (◆)NH₄Cl above a background concentration of 200 mM sodium phosphate. Data taken from Leodidis and Hatton (1989b).
follows the same functionality with ion type as did micelle size. In other words, ions that stabilized larger micelles also had the highest transference of protein.

The implications of this work are that the protein partitioning could be controlled by properly selecting the types of ions, and that this might even be used to select between proteins of like charge but different size. For forward transfer, it would be desirable to use ions in the aqueous phase which would promote larger water pool formation in the absence of protein. For example, with the AOT/isooctane system, the choice of ion would be $\text{Na}^+ > \text{Ca}^{2+} > \text{Rb}^+ > \text{Cs}^+$. Reversing the order would help promote efficient back transfer.

In regard to our work, this combination of effects may prove to be quite interesting. In all of the previous studies on the role of a protein's charge on partitioning, pH changes have been used to change the protein charge. Accompanied with these pH changes can be changes in the relative concentrations of different ions in solution. In light of the above work, changing the pH of the aqueous phase may very well result in a change in the water pool size, which then might require greater charge interactions for effective partitioning. In our research, charge manipulation is controlled by modifying the protein, not by changing the pH. This should allow us to probe the role of charge without the competing effects of ion concentration and type.

**Summary** Factors which have the largest effect on protein partitioning are those that would be expected from any charge based process. The strength of the interaction between a protein and a reversed micelle will depend, first of all, on the sign and magnitude of their respective charges. For a given protein, the sign and magnitude of its charge is determined by the pH of the medium. The surfactant’s head group determines the nature of the charge of the reverse micelle. These two factors will determine whether there will be any interaction at all.

The magnitude of the charge interaction necessary for protein solubilization is contingent upon many things. Foremost among these is the stability of the micelle. Certain
surfactants do not form large micelles; others do, but are bounded by absolute limits. If the protein to be solubilized is larger than the stable empty micelle size, larger interactions need to occur for the protein to partition. The charge distribution also plays a role — an asymmetric distribution on the protein allows for a lower difference between the pH of extraction and the protein's pI than for a highly symmetric distribution.

Additionally, the ionic strength of the aqueous phase affects the interaction through charge screening. The ions reduce the repulsion between surfactant head groups, allowing more compact micelles to form. The ions also screen between the charged residues on the protein and the surfactant head groups, reducing the effective charge difference. For a given potential difference to be maintained, the charge on the protein must be higher. A third factor is the type of ions in the aqueous phase. Certain types of ions stabilize larger or smaller micelles, and so the level of charge interaction depends on the ion type.

All in all, there are factors that are reasonably straightforward (sign and magnitude of the charge on the protein and the surfactant head group) and factors that are interconnected (micelle stability as influenced by the surfactant type, ionic strength of the aqueous phase, and the types of ions in the aqueous phase).

**Strategies for Enhancing Selectivity**

There have been essentially two methods used to enhance the selectivity of reversed micellar extraction towards a particular protein. One such method is covered in this dissertation, that of modifying the protein of interest using charged fusions to increase the electrostatic interactions between the protein and the reversed micelle. A second method has been used by Kelley *et al.* (1993), in which the authors use affinity cosurfactants created by attaching ligands to alkyl groups to aid in increasing selectivity. In their paper, Kelley *et al.* used octyl glucoside, lecithin, and hexyl boronic acid as affinity cosurfactants for concanavalin-A, myelin basic protein, and α-chymotrypsin, respectively. The primary
surfactant was AOT because of its ability to form relatively large micelles and to tolerate cosurfactants. Representative results are shown in Figure 8, in which concanavalin-A transfer is plotted as a function of the water uptake number. As a reminder, the water uptake number is influenced by such things as ionic strength and ion type, and what is necessary for solubilization in cases of small water uptake is substantial interaction between the protein and the reversed micelle. The shift in the solubilization curve to the left, at smaller water uptake numbers, indicate that the interaction between the affinity cosurfactant and the protein, in combination with the electrostatic interactions between the primary surfactant and the protein, is stronger than that provided by electrostatic contributions alone.

The results from this study show that reversed micelles may be used to selectively extract proteins over a wide range of salt and pH conditions through the use of affinity cosurfactants. There are limitations to this technique, some of which are tradeoffs between high recovery and selectivity, as well as dependence on the binding strength of the ligand with the protein. The best results in this study were provided by ligands that had dissociation constants on the order of $1 \times 10^{-6}$ M.

**Genetic Modifications to Enhance Protein Recovery**

A number of different systems have been developed that have attempted to use genetic engineering techniques to ease the recovery of the proteins from fermentation broth. Such systems have included fusing whole enzymes, such as $\beta$-galactosidase, polypeptide-binding proteins, carbohydrate-binding domains, antigenic epitopes and poly(amino acids) to the target protein (Ford *et al.*, 1991). These fusions ranged in size from 1 amino acid to 116 000 MW fragments, and have been used with a wide selection of purification methods including binding to immobilized substrate and antibodies, ion exchange, and precipitation (Ford *et al.*, 1991).

One strategy that has been used with much success in the purification of proteins using
Figure 8. Transfer curve for concanavalin-A showing the effect of affinity cosurfactant octyl glucoside on partitioning. Control reversed micelles contain AOT only. Data taken from Kelley et al. (1993).
relatively inexpensive recovery methods is using fusions of charged amino acids. Charged polypeptides consisting of either poly(aspartic acid) or poly(arginine) fused to both β-galactosidase and glucoamylase have been used in polyelectrolyte precipitation (Parker et al., 1990; Suominen et al., 1993), ion exchange chromatography and ion exchange membranes (Heng and Glatz, 1993; Stafslien et al., 1994), and aqueous two phase extraction (Luther and Glatz, 1994).

**Fusions to Glucoamylase**

Glucoamylase from *Aspergillus awamori* is a monomeric enzyme that catalyzes the hydrolysis of β-D-glucose from the non-reducing end of starch molecules by cleaving α-(1→4) and α-(1→6) glycoside bonds. Glucoamylase exists in two forms, GAI and GAI, which differ in their ability to hydrolyze raw starch. The difference in the primary structure of the two enzymes is that of an approximately 17 kDa carboxyl-tail region, present in GAI but absent in GAI (Sierks, 1988). This region, which includes amino acids 513 to 616, is believed to be removed by limited proteolysis of GAI (Svensson et al., 1986). The functionality of this region is in the binding of the enzyme to raw starch.

The *Aspergillus awamori* gene for glucoamylase has been cloned as a cDNA fragment and is expressed in *Saccharomyces cerevisiae*, where it is secreted (Innis et al., 1985). Specific activity and expression are not affected for the GA'CD series, though expression of the GA'ND mutants is hindered with increasing tail length (Suominen et al., 1993). Tail integrity was confirmed using isoelectric focusing (Suominen et al., 1993) and through non-denaturing polyacrylamide gel electrophoresis (Deborah Stachon, unpublished results).

The three dimensional structure of a truncated form of glucoamylase is known (Aleshin et al., 1992), and has been used to determine which charged residues are available for interaction with the solvent in order to estimate the net charge on the protein (see the Appendix for the charge estimation). Glucoamylase has been modified to include charged poly(amo
acid) residues at both the carboxyl and amino termini (Suominen et al., 1993), and was subsequently modified in this study as described in Paper 1. The genetic sequences for the mutants are shown in Figure 9.
Wild type:

ss-Ala-Asn-Val-Ile-Ser-Lys-Arg-Ala-Thr...
3'--... AAT GTC ATT TCC AAG CGC GCG ACC...

GA'ND5

ss-Ala-Asn-Val-Ile-Ser-Lys-Arg-Asp-Tyr-Asp-Asp-Asp-Asp-Lys-Ala-Arg-Ala-Thr...
3'--... AAT GTC ATT TCC AAG CGC GAC TAC GAC GAC GAT GAC AAG GCG CGC GCG ACC...

GA'ND10

3'--... AAT GTC ATT TCC AAG CGC GAC TAC GAC GAC GCT GAC GAC GAC GAT GAC GAC AAG GCG CGC GCG ACC...

GA'CD0

...-Met-Ala-Tyr-***
3'--... GATCC ATG GCA TAC TAG A AGCTT G...5'

GA'CD5

...-Met-Ala-Asp-Asp-Asp-Asp-Asp-Tyr-***
3'--... GATCC ATG GCA GAC GAC GAT GAT GAT TAC TAG A AGCTT G...5'

GA'CD10

3'--... GATCC ATG GCA GAC GAC GAT GAC GAT GAT GAT GAT GAT GAT TAC TAG A AGCTT G...5'

Figure 9. Nucleotide and amino acid sequences of fusions to Aspergillus glucoamylase (Suominen et al., 1993).

*** denotes a translation stop codon
REVERSED MICELLAR EXTRACTION OF CHARGED FUSION PROTEINS

A manuscript accepted for publication by Biotechnology Progress

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Summary

We have investigated the use of charged fusion tails with the enzyme glucoamylase in reversed micellar extraction. The addition of the charged tails increased the fraction of enzymatically active protein recovered at a given pH, with the tails containing the largest number of charges being recovered at the highest level. The series of mutations also allows for investigation of the charge-dependent behavior of reversed micellar extraction. However, in this case, the change in protein charge via fusions had a lesser impact than did the change in charge via a pH change. The difference may be due to the difficulty of partitioning the hydrodynamically larger fusion protein.

Introduction

The separation and purification of protein products from fermentation broth for use in the pharmaceutical, food, or consumer product industries are quite complex. This complexity is due to the nature of the broth: it is dilute, yet it contains a wide variety of compounds, ranging from proteins to nucleic acids, lipids and metabolites. The need for high-purity of the protein products, especially for pharmaceutical use, requires that separation steps of high specificity be used in the purification scheme.

Separation steps differ in their specificity towards a given protein of interest as well as in their relative cost. Steps with high selectivity, such as affinity chromatography or immuno-affinity precipitation, are generally accompanied by high cost, whereas processes with low specificity, such as ammonium sulfate precipitation, are correspondingly cheaper (Bisbee,
One way to make a separation process with low selectivity more selective is to modify the protein of interest to confer upon it unique characteristics that allow separation to occur. One such modification is to use so-called "purification fusions". These fusions consist of amino acid sequences or even entire regions of other proteins genetically added to the protein of interest. This technique has been used to add regions conferring immunoaffinity, substrate affinity, metal chelation and high charge density on proteins (Ford et al., 1991).

While most of these regions are appropriate for relatively expensive methods of purification, fusions with high charge density have been used in the purification of β-galactosidase using polyelectrolyte precipitation (Parker et al., 1990) and hollow fiber ion exchange membranes (Heng and Glatz, 1993).

One purification method that has the potential for the large-scale separation of protein products is reverse micellar extraction. Reverse micellar extraction takes advantage of the tendency of some surfactants to aggregate about pools of water while in organic solvents. Protein molecules may be solubilized inside of these water pools, with the factors determining the strength of the partitioning being charge on the protein (Göklen and Hatton, 1987), the ionic strength of the aqueous solution (Göklen and Hatton, 1985; Dekker et al., 1987), the type of ions in solution (Leodidis and Hatton, 1989), and the size of the protein as well as its charge asymmetry (Wolbert et al., 1989).

Charged fusion proteins might be useful in reversed micellar extraction for several reasons. They may be used to increase the electrostatic interactions between the protein and the reverse micelle without resorting to pH extremes for large proteins. An additional area in which the fusions may play a role is in their high charge density providing a highly asymmetric charge distribution, giving an effect analogous to the phenomena reported by Wolbert et al. (1989). Something that may confound this strategy is that the high charge density would lead to an extended conformation for the tail. An extended conformation has been observed with
charged fusions to β-galactosidase (Niederauer et al., 1993), where charged fusions alter the retention time of the protein on a size exclusion column. The resultant increase in hydrodynamic radius would require a larger charge interaction for solubilization to occur. It is evident from Wolbert et al. (1989) that proteins with a higher molecular weight (and thus increased volume) require a higher difference between the pH of the solution and the pI of the protein (a higher net charge on the protein) for solubilization to occur than small proteins.

In order to test this strategy, we have studied the behavior of charged fusion proteins in reverse micellar extraction, using the enzyme glucoamylase from Aspergillus awamori expressed in the yeast Saccharomyces cerevisiae. Glucoamylase is a protein with a molecular weight of approximately 65,000. Because of its relatively large size, a large difference between the pH of the forward extraction phase and the pI of the protein would normally be expected for appreciable transfer to occur (Wolbert et al., 1989). The surfactant system is trioctylmethyl ammonium chloride/(nonylphenoxy) pentaethylene oxide/1-octanol in isooctane.

Materials and Methods

Strains and Constructions

The Saccharomyces cerevisiae strain C468 with the 2-μm vector pGAC9, producing the enzyme glucoamylase from the plasmid, was manipulated to produce six mutant versions of the enzyme. The gene for glucoamylase was a gift from the Cetus Corporation (Emeryville, CA) and was modified by fusing oligonucleotides on the 5' and 3' ends of the gene using standard procedures (Maniatis et al., 1982). These oligonucleotides coded for fusion tails of the following form. For the amino-terminal fusions, the sequence is Asp-Tyr-(Asp)_n-Lys-Ala-Arg, with n = 4 and 9 for GAND5 and GAND10, respectively (Suominen et al., 1993). The carboxyl-terminal fusions were made on a truncated form (482 amino acids) of the enzyme (first constructed by Evans et al. (1990)) and have the sequence Met-Ala-(Asp)_n-Tyr with n = 0, 5, and 10 for GACD0, GACD5, and GACD10, respectively (Suominen et al., 1993).
amino-terminal fusions were subsequently modified to have the same base 482 amino acids as the carboxyl-terminal fusions by splicing the amino-terminal end of the GANDx genes with the carboxyl-terminal end of GACDO. These mutants are designated GA'ND5 and GA'ND10. The designations for the carboxyl-terminal fusions GACDx have been changed to GA'CDx to denote that both the GA'NDx and GA'CDx mutants are based on the same truncated (482 amino acids) gene. Finally, an additional mutant with the sequences Asp-Tyr-(Asp)4-Lys-Ala-Arg at the amino terminus and Met-Ala-(Asp)5-Tyr at the carboxyl terminus, designated GA'ND5CD5, was constructed by splicing the amino-terminal region of GAND5 with the carboxyl-terminal end of GA'CD5. See Figure 1.

Cells were grown in yeast SD minimal media (Sherman et al., 1983) supplemented with 100 mg/L L-histidine using 2% glucose as a carbon source. Overnight cultures were grown from single colonies picked from SD+His agar plates, and these were in turn used to inoculate fermentation vessels. The cells were grown in either 15- or 50-L fermentors at the ISU Fermentation Facility at 30°C, pH 4.5, and 80% dissolved oxygen. Glucose was added periodically to restore its concentration to 2%. Cells were harvested at 5–7 days. In this expression system, glucoamylase is secreted, so that the cells were separated from the fermentation broth by passing the cell-containing broth through a hollow fiber microfiltration apparatus with a 0.1-μm nominal pore size (Amicon, Danvers, MA).

**Purification of Glucoamylase**

The cell broth was first concentrated using a spiral wound hollow fiber apparatus with a 10 000 nominal molecular weight cutoff (Amicon, Danvers, MA). This same device was used to diafilter the broth with a 100 mM sodium acetate solution (pH 4.5) containing 500 mM sodium chloride. This concentrated solution was passed over an acarbose affinity column prepared according to Clarke and Svensson (1984). The bound protein was washed with 100 mM sodium acetate/500 mM sodium chloride (pH 4.5) and eluted with 1.7M Tris-HCl.
Figure 1. Schematic diagram indicating alterations to the glucoamylase gene from that described by Suominen et al. (1993) in order to construct the GA'ND and GA'NDCD fusions.
(pH 7.6). Collected samples were dialyzed overnight in Spectra Por 3 dialysis tubing (3500 MW cutoff) versus 10 mM sodium acetate (pH 4.5).

Glucoamylase Activity Assay

Activity assays of glucoamylase were performed using a modified procedure from Svensson et al. (1982). One activity unit is defined as the amount of glucoamylase required to release 1 μmol of glucose from soluble starch in 1 min. One hundred microliters of sample was added to 2 mL of a 2% soluble starch solution in 50 mM sodium acetate (pH 4.5). Three hundred-microliter aliquots were drawn off at desired intervals, and the reaction was stopped by pipetting the aliquots into tubes containing 200 mL of 2.5M Tris-HCl (pH 7.0). Glucose concentration was determined using the method of Banks and Greenwood (1971) using a Tris-HCl/glycerol buffer.

Reversed Micellar Extraction

The reverse micellar extraction system used was based on that of Dekker et al. (1987a). The system consisted of a 0.4% (w/v) solution of the cationic surfactant trioctylmethyl ammonium chloride (TOMAC) (Eastman Kodak, Rochester, NY) with 0.088% (w/v) (nonylphenoxy) pentaethylene oxide (Rewopal HV5) (generously provided by Rewo Chemische Werke GMBH, Steinan an der Strasse, Germany). The cosurfactant used was 1-octanol (0.1% (v/v)), and the continuous phase was isooctane (Fisher Scientific, Pittsburgh, PA). The aqueous-phase electrolyte for forward extraction was 50 mM ethylenediamine, titrated to the appropriate pH using HCl. For back extraction, 100 mM sodium acetate (pH 4.5) with 500 mM sodium chloride was used.

Two hundred microliters each of aqueous and organic phases were mixed in microcentrifuge tubes by vortexing. The phases were allowed to separate by settling. One hundred and fifty microliters of the organic phase was removed and added to a separate microcentrifuge tube containing an equal amount of back-extraction buffer. Mixing was again
provided by vortexing, and the phases were allowed to separate. The organic phases were removed, and the aqueous phases from the forward and back extractions, as well as the stock solutions, were assayed according to the procedure described above.

**Size Exclusion Chromatography**

Gel permeation chromatography studies were performed using a Phenomenex (Torrance, CA) Biosep-SEC-S4000 column on an ISCO (Lincoln, NE) HPLC system. The continuous phase was 100 mM potassium phosphate buffer (pH 7.0). Protein was detected by absorbance at 280 nm (Beckman Model 165 variable wavelength UV detector, Fullerton, CA).

**Results and Discussion**

The partitioning results will be discussed from two perspectives: the fractional recovery of protein in reversed micellar extraction and the charge-dependent behavior of proteins in reversed micellar extraction. The first is of interest as a strategy for integrating biological and separation-based approaches to selective product recovery. The second perspective is important because fusions could be used to elucidate the role of charge in the solubilization of proteins in reversed micelles. We have changed the charge on the individual molecules by adding charged residues rather than by changing pH (which has accompanying changes in external electrolyte composition and concentration), and this could better isolate the effects of protein charge.

**Fractional Recovery**

As shown in Figure 2, the mutants with the highest number of additional charges, GA'ND10, GA'CD10, and GA'ND5CD5, partitioned more strongly than the mutants with fewer additional charges. This is consistent with the finding that more highly charged proteins partition more strongly (Wolbert *et al.*, 1989). These results support the strategy outlined in the Introduction, that of adding charged fusion tails to proteins so that recovery can be
Figure 2. Fraction of initial activity recovered in the back-extraction buffer as a function of the pH of forward-extraction buffer for carboxy-terminal tails (a) and amino-terminal tails (b). Back-extraction conditions were constant.
achieved in a more moderate pH range. The results are also in agreement with those of Parker et al. (1990), Heng and Glatz (1993), and Suominen et al. (1993), who found that enzymes with the highest number of additional charges were separated most strongly using either polyelectrolyte precipitation or ion exchange.

Comparison of the values reported here for the fraction recovered with those reported by Göklen and Hatton (1985) might lead one to believe that the strategy of altering the protein to enhance recovery is of little utility; after all, the maximum amount recovered here is less than 40% compared to near quantitative results achieved by Göklen and Hatton with cytochrome c. However, the amounts reported here are encouraging when viewed with reference to the size of the protein. Wolbert et al. (1989) experimented with two proteins of sizes similar to glucoamylase: alcohol dehydrogenase and bovine serum albumin. Alcohol dehydrogenase had a recovery of 40% at a difference between the pH of extraction and its pI of 6.1 units. BSA was not transferred at all. In contrast, GA'ND10, GA'CD10, and GA'ND5CD5 were recovered at levels between 20 and 35% at a difference between the pI and the pH of extraction of approximately 4.5 units. At low pH's, the balance of the activity is found in the remaining forward-extraction buffer, while at higher pH's the activity balance did not close. Glucoamylase added to forward-extraction buffer which had been preequilibrated with the organic phase showed the same pH-dependent inactivation pattern, indicating that trace amounts of the organic phase in the forward-extraction buffer is the cause of inactivation. TOMAC is likely responsible as a similar effect was reported by Dekker et al. (1989) with α-amylase. The location of the inactivated glucoamylase could not be determined under the conditions we were using because of interference with both the Bradford and BCA protein assays, as well as with absorbance at 280 nm.
Charge Based Interactions

The charges on the proteins were estimated by using the Henderson-Hasselbalch equation using pK values for amino acids from a variety of sources. When the fraction of activity recovered is plotted against estimated net charge instead of aqueous phase pH, as in Figure 3, we see some interesting trends. The first is that the net charge at which the activity recovered increases is not the same for the different mutants. Secondly, the upswings for each of the mutant classes (0, 5, or 10 additional residues) are distinct. Since the size (mass) difference among the mutants is at most 3.5%, one would expect that the behavior would be nearly identical when plotted as a function of charge. This is not the case. There would appear to be two different explanations for this behavior. The first is that the fusion tails are not completely ionized because of the high charge density, and the resultant estimated charge is inaccurate. A different explanation is that even though the fusions are small in relation to the total number of amino acids in the proteins, the presence of the highly charged region leads to an extended conformation and, thus, an expanded hydrodynamic radius that would hinder extraction.

Characterization of several of the mutants using a size exclusion column is seen in Figure 4. Retention time decreased with an increasing number of residues. GA'CD0 had an apparent molecular weight of 67,000 while GA'CD10 had an apparent molecular weight of 74,000. This increase in apparent molecular weight is much larger than would be expected from simple changes in the actual mass of the protein and is likely indicative of the fused peptide having an extended conformation. The correlation from Wolbert (1989) can be used to estimate the required increase in the difference between the optimal pH of extraction and the pI of the protein to compensate for the larger size. For this increase of 7000, the required pH difference should increase approximately 0.7 pH units. On the basis of pI values from the charge estimates, the difference between the pH at which the upswings occurred in Figure 3 and
Figure 3. Fraction of initial activity recovered in the back-extraction buffer as a function of the estimated net charge on the protein in the forward-extraction buffer. Back-extraction conditions were constant, while the pH of the forward extraction was varied from 4.75 to 8.75.
Figure 4. Representative size exclusion chromatogram indicating the size differences between the mutants (one of three replicates).
the pI of these mutants also increases approximately 0.7 pH units. Thus the failure of all fusions to lie on a single curve in Figure 3 can be accounted for by the increase in hydrodynamic size with addition of the tail.

Conclusions

The fusion proteins with the greatest number of additional charges are partitioned more strongly at a given pH than either the control version or those with fewer additional charges. The behavior is not well explained by charge alone, as it appears that the benefits of increased charge are partially offset by increased size of a fusion protein carrying an extended tail. Inactivation by organic-phase components limits the percent active enzyme that may be recovered.

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EXTRACTION OF CHARGED FUSION PROTEINS IN REVERSED MICELLES: COMPARISON BETWEEN DIFFERENT SURFACTANT SYSTEMS

A paper to be submitted for publication in Biotechnology Progress

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Abstract

Behavior of a series of fusion proteins of varying charge in reversed micellar extraction was studied. The proteins consisted of the enzyme glucoamylase from Aspergillus awamori joined to short peptides containing from 0–10 additional aspartate residues. The fusions were partitioned into two different cationic surfactant systems, one based on the surfactant trioctyl methyl ammonium chloride (TOMAC) and the other on cetyl trimethyl ammonium bromide (CTAB). These two systems differed chiefly in micelle size as measured by the surfactant to water ratio, \( W_Q \). Water numbers were determined for the TOMAC system, with values of approximately 10, and as a function of pH and ionic strength for CTAB for each of the mutant enzymes. For the CTAB system, water numbers were as low as 50 with NaCl concentrations of 500 mM and as high as 68 at 300 mM NaCl. The enzyme partitioned most strongly using CTAB, with maximal recoveries approaching 95%. However, in the CTAB system there were no significant differences in behavior between the mutants because of the relatively large micellar size, even under high salt concentrations. Extraction of the control enzyme from clarified cell broth indicated that broth components did not interfere with partitioning.

Introduction

There has been much interest in the recovery of biological products from fermentation broths, with the primary features of interest being the cost of the separation process and the
purity and yield of the recovered product. As a result, many different types of separation processes have been developed, each attempting to provide unique advantages in speed, specificity, volume reduction, etc. One method that has recently been the source of increasing interest is reversed micellar extraction, which employs surfactants to solubilize proteins and water in an organic medium. The properties of the surfactants in the reversed micelles, particularly the charge associated with the headgroups, play an especially important role: the driving force for extraction is the electrostatic interaction between the charged surfactant headgroups of the micelle and the charged residues on the protein, mediated by a size exclusion effect (Wolbert et al., 1989). As a result, the environmental factors that affect the efficiency of the extraction are the pH (Göklen and Hatton, 1987) and ionic strength of the aqueous phase (Göklen and Hatton, 1985; Dekker et al., 1987b) along with the type of ions in solution (Leodidis and Hatton, 1989), the surfactant and the organic phase constituents (Krei and Hustedt, 1992).

As recently reviewed (Kelley et al., 1993), reversed micellar extraction is not without its problems, as surfactant reuse has not been addressed, recovery is generally below 80%, and overall purification factors are often low. Because of the last problem, it has become necessary to devise a method for increasing the specificity of the selection mechanism. Two such methods have recently been suggested. The first involves the use of affinity cosurfactants, in which ligands specific for the proteins of interest are covalently attached to alkyl groups of various lengths. Such ligands have been used to selectively extract several different proteins, including concanavalin A, myelin basic protein, and chymotrypsin (Kelley et al., 1993). A second approach has been to utilize genetic engineering techniques to modify the protein of interest to include additional charged residues in the form of a charged purification fusion, a procedure which has been used to extract the enzyme glucoamylase (Forney and Glatz, 1994).
One of the key factors that determines protein partitioning into a reversed micellar phase is the size of the protein. It is evident from Wolbert et al. (1989) that as protein size increases, it becomes much more difficult to partition the protein into the reversed micelle. The explanation is that a larger protein requires a reversed micelle larger than what would be thermodynamically stable based solely on surface tension considerations. One could attempt to get around this problem by altering conditions so that the normal size of the reversed micelles would be larger through manipulation of the cosurfactants or the organic continuous phase or the like. A recent paper by Krei and Hustedt (1992) explored several different cationic surfactant systems, reporting water number ($w_q$) measurements. From their data, it is apparent that the CTAB surfactant stabilizes much larger reversed micelles than the TOMAC surfactant. In view of this, we experimented with CTAB in an attempt to improve on the low recoveries of glucoamylase realized using TOMAC (Forney and Glatz, 1994).

The purpose of this study is to investigate the role of charged peptide fusions on the extraction of glucoamylase in different surfactant systems. Variables considered are extraction temperature and the pH and ionic strength of the aqueous solution. Two different surfactant systems are tested: trioctyl methyl ammonium chloride (TOMAC)/ (nonylphenoxy) pentaethyline oxide/1-octanol/isoctane and cetyl trimethyl ammonium bromide (CTAB)/hexanol/butanol/isoctane.

**Materials and Methods**

Glucose oxidase, horseradish peroxidase, and o-dianosine dihydrochloride were purchased from the Sigma Chemical Company (St. Louis, MO). All other reagents, unless otherwise noted, were purchased from Fisher Scientific (Pittsburgh, PA).

**Strains and Constructions**

The *Saccharomyces cerevisiae* strain C468 with the 2-μm vector pGAC9, producing the enzyme glucoamylase from the plasmid, was manipulated to produce six mutant versions of
the enzyme. The gene for glucoamylase was a gift from the Cetus Corporation (Emeryville, CA) and was modified by fusing oligonucleotides on the 5' and 3' ends of the gene using standard procedures (Maniatis et al., 1982). The mutants used in this study have charged fusions on the carboxyl termini. These mutants are based on a truncated form (482 amino acids) of the enzyme (first constructed by Evans et al. (1990)) and have the sequence Met-Ala-(Asp)ₙ Tyr with ƞ = 0, 5 and 10 for GA'CD0, GA'CD5, and GA'CD10, respectively (Suominen et al., 1993).

Cells were grown in yeast SD minimal media (Sherman et al., 1983) supplemented with 100 mg/L L-histidine using 2% (w/v) glucose as a carbon source. Overnight cultures were grown from single colonies picked from SD+His agar plates, and these were in turn used to inoculate fermentation vessels. The cells were grown in 50-L fermentors at the ISU Fermentation Facility at 30°C, pH 4.5, at 80% dissolved oxygen. Glucose was added periodically to restore its concentration to 2%. Cells were harvested at five to seven days. In this expression system, glucoamylase is secreted, so the cells were separated from the fermentation broth by passing the cell-containing broth through a hollow fiber microfiltration apparatus with a 0.1 μm nominal pore size (Amicon, Danvers, MA).

Purification of Glucoamylase

The cell broth was first concentrated using a spiral wound hollow fiber apparatus with a 10 000 nominal molecular weight cutoff (Amicon, Danvers, MA) and then diafiltered with water. This feed solution was diluted 1:1 with 50 mM sodium acetate and adjusted to pH 6.0. The solution was loaded onto a 50 mm O.D. QUAT Acti-Disk™ Separation and Purification Cartridge (FMC BioProducts, Rockland, ME), washed with a solution of 50 mM sodium acetate, pH 4.5 with 100 mM sodium chloride, and eluted with 50 mM sodium acetate (pH 4.5) with 500 mM sodium chloride (Stafslien et al., 1994). Collected samples
were dialyzed overnight in Spectra Por 3 dialysis tubing (3500 MW cutoff) versus 10 mM sodium acetate (pH 4.5).

**Glucoamylase Activity Assays**

Activity assays were performed by determining the rate of glucose liberation from soluble starch (Forney and Glatz, 1994). One hundred microliters of sample was added to 2 mL of a 2% (w/v) soluble starch solution in 50 mM sodium acetate, pH 4.5. Three hundred microliter aliquots were drawn off at desired intervals, and the reaction was stopped by pipeting the aliquots into tubes containing 200 μL of 2.5M Tris/HCl, pH 7.0. Glucose concentration was determined using the method of Banks and Greenwood (1971) using a Tris-HCl/glycerol buffer. One activity unit is defined as the amount of glucoamylase required to release one micromole of glucose from soluble starch in one minute.

**Reversed Micellar Extraction**

Two surfactant systems were investigated. The first consisted of 200 mM CTAB in a solution of isooctane/hexanol (ratio 19:1) with 10% (v/v) butanol. The second system consisted of a 0.4% (w/v) solution of the cationic surfactant TOMAC (Eastman Kodak, Rochester, NY) with 0.088% (w/v) (nonylphenoxy) pentaethylen oxide (Rewopal HV5) (generously provided by Rewo Chemische Werke GMBH, Steinau an der Strasse, Germany). The cosurfactant used was 1-octanol (0.1% (v/v)), with isooctane as the continuous phase.

For the CTAB system, the aqueous phase electrolyte for forward extraction was a solution of 10 mM sodium phosphate at a given pH with 100–500 mM sodium chloride. In the case of the TOMAC system, the aqueous phase electrolyte was 50 mM ethylene diamine, titrated to the appropriate pH using HCl. Unless otherwise stated, protein concentration was approximately 25 μg/mL. For both systems, the back-extraction buffer was 50 mM sodium acetate (pH 4.5) with 500 mM sodium chloride.
Three hundred fifty microliters each of aqueous and organic phases were mixed in microcentrifuge tubes at room temperature by vortexing. For the CTAB system, phase separation was accomplished by centrifugation in a Fisher model 235 microcentrifuge (13,600 \( x \ g \)) for 45 min., while in the TOMAC system the phases separate completely by settling. Two hundred fifty microliters of the organic phase was removed and added to a separate microcentrifuge tube containing an equal amount of back-extraction buffer. Mixing was again provided by vortexing at room temperature. The CTAB back-extraction was either immediately centrifuged for 15 min. at room temperature for phase separation or allowed to incubate at 35°C for 15 min. followed by centrifugation (at room temperature) for 15 min. The TOMAC back-extraction was incubated at 35°C for 15 minutes, by which time the phases had separated. The organic phases were removed, and the aqueous phases from the forward and back-extraction as well as the stock solutions were assayed for glucoamylase activity.

**Karl Fischer Titration**

The water content of the reversed micelles was determined by Karl Fischer titration using a Fisher Scientific Computer Aided Titrimeter. Titrant was Riedel-de Haën Hydranal-Composite/5K, and the continuous phase was HPLC grade methanol. Samples were introduced into an airtight titration vessel via injection using a Hamilton syringe and titrated to an endpoint of \(-100 \text{ mV}\) with a persistence of 10 seconds (Anonymous, 1987).

**Results and Discussion**

Figure 1 shows the fraction of the initial glucoamylase activity recovered in the back-extraction buffer from a CTAB reversed micellar phase as a function of the fusion tail length and the pH of the forward-extraction buffer. There are several things to note from this figure. The first is that the recovery is virtually independent of both the pH and the additional charged residues. This is in direct contrast to the results reported in Forney and Glatz (1994),
Figure 1. Fraction of initial activity recovered in the back-extraction buffer from a CTAB reversed micellar phase as a function of the pH of the forward-extraction buffer. Back extraction was achieved by mixing 50 mM sodium acetate (pH 4.5) with 500 mM sodium chloride with the reversed micellar phase at room temperature. Error bars represent one standard deviation.
where recovery was dependent on both the pH and the fusion tail length with the same fusions and over the same pH range. Furthermore, the activity balance did not close, with no activity remaining in the forward-extraction buffer under these conditions, causing one to wonder if either this surfactant/enzyme combination displayed inactivation behavior that was not pH dependent (as was the inactivation in Forney and Glatz, 1994) or if glucoamylase remained in the organic phase and an alternative recovery method would be necessary. The second important feature of this figure and the figures to follow is the high degree of variability in the fraction of activities recovered, as compared to that reported later with the TOMAC system and in Forney and Glatz (1994). The error bars shown represent one standard deviation, signifying large variations in the values measured. This variability was particularly high from day to day, and lower for a series of extractions conducted on a single day. Potential sources of the variability are fluctuations in room temperature and/or centrifuge rotor temperature. Because of this, we looked at behavior as a function of a system variable only on experiments within a day.

There have been several recent attempts to utilize alternative recovery methods from the organic phase, including addition of dewatering agents such as isopropyl alcohol (Carlson and Nagarajan, 1992), formation of clathrate hydrates via pressurization (Phillips et al., 1991), adsorption onto silica (Leser et al., 1993) and temperature shifts (Dekker et al., 1991). The last of these, high temperature (35°C) incubation, was used by Dekker et al. (1991) to remove α-amylase from a TOMAC system in a single step. In that case, the authors removed the reversed micellar phase after forward extraction and raised the temperature above 35°C, which caused the reversed micelles to dewater, thus releasing the solubilized protein. However, in this study raising the temperature of the organic phase to 35°C did not cause protein release. Nonetheless, incubation of the organic phase with the back-extraction buffer at 35°C did increase the amount of activity recovered from roughly 40% to 90% (Figure 2).
Figure 2. Fraction of initial activity recovered in the back-extraction buffer from a CTAB reversed micellar phase as a function of the pH of the forward-extraction buffer. Back-extraction conditions were as in Figure 1 followed by an incubation of the mixed phases at 35°C. Error bars represent one standard deviation.
The shape of the curve in Figure 2 is typical for reversed micellar extraction, with increasing solubilization with increasing pH until a value is reached at which partitioning is more or less constant. It is at this point at which electrostatic interactions are more than sufficient to drive solubilization of the protein, and one would not expect much dependence on protein net charge as the charge is increased further. There are a number of points about this figure deserving comment. The first is that, as in Figure 1, there is no pH dependence for pH values greater than 5.5, but here we have added lower pH values and thus see there the expected dependence. Secondly, in Figure 2 we see much less variability at the high pH levels than we did in Figure 1. This is not only because we are looking at multiple points from one set in a day, whereas Figure 1 consisted of experiments from different days, but is also likely due to the fact that the incubation at 35°C gave a very consistent temperature during back extraction as opposed to the less regulated room and rotor temperatures to which the samples of Figure 1 were exposed. The next thing to note is that there do not appear to be any significant difference between the partitioning of the mutants, even at the low pH values. It would be expected that if a difference in partitioning would be apparent, it would be at low pH values, where the charge differences between the mutants would be largest on a percentage basis. For example, at pH 4.5, the estimated net charge on GA‘CD0 is -8.5, whereas the charge on GA‘CD10 is 96% larger, at -16.6. In this region where the partitioning is still increasing with increasing charge, one would expect that a doubling of the charge would lead to a much larger difference in partitioning than what is observed. In the TOMAC system, we attributed this discrepancy to partitioning a hydrodynamically larger fusion protein into a relatively small micelle, but that likely is not true in this case as the micelles are so large to begin with. Finally, the difference between the pH of “optimal” extraction and the pI of the protein is quite low relative to that of the TOMAC system. Here we see a difference of 1.5 to 1.8 pH units, much smaller than the 4.5 pH units reported by Forney and Glatz (1994) with the TOMAC system or the 6 pH
units expected from Wolbert et al. (1989). This signifies an ease of solubilizing the relatively large protein relative to TOMAC, presumably due to the larger size of the reversed micelles.

One further check on the electrostatic interaction dependence of partitioning would be to increase the salt concentration of the forward-extraction aqueous phase, thus decreasing the reversed micellar size and also the charge interaction between the micelle and the protein. This is shown in Figure 3, where we see two trends. The first is that partitioning decreases with increasing salt concentration, supporting the traditional view of charge-dependent partitioning. The second is that again there are no significant differences between the mutants, again supporting the idea that as long as the charge is favorable in sign, the determining factor in the partitioning may be other than the magnitude of the charge.

Because of the success in increasing the recovery from CTAB reversed micelles by elevating the temperature of the back-extraction step, we went back to our original TOMAC system in which we saw the difference in behavior between the mutants but low recoveries. In this case, elevated temperature incubation did not yield any differences in recovery, with the amount of active enzyme recovered being consistent in magnitude with that reported in Forney and Glatz (1994). Here as in the previous paper, maximal recoveries were on the order of 20 to 30%, depending on the particular mutant, and occurred at pH values near 7.25. The results from these experiments are shown in Figure 4. While there were large variations in the fraction of activity recovered in the CTAB system, the amounts recovered with TOMAC show very little variability. Error bars in Figure 4 do not represent one standard deviation as in Figures 1–3, but instead represent a 95% confidence level based on a pooled standard deviation. Also, the values are consistent not only from day to day but also from week to week, indicating that this system does not appear to be as dependent on environmental factors as CTAB. As is the case reported by Dekker et al. (1989), the decrease in recovery at higher pH values (pH > 7.5) is from inactivation.
Figure 3. Fraction of initial activity recovered in the back-extraction buffer from a CTAB reversed micellar phase as a function of both the pH and the NaCl concentration of the forward-extraction buffer. Symbols for GA'CD0 (100/300/500 mM NaCl) ■/□/□, GA'CD5 ●/○/○, and GA'CD10 ▲/▲/▼. Back extraction conditions were as in Figure 2.
Figure 4. Fraction of initial activity recovered in the back-extraction buffer from a TOMAC reversed micellar phase as a function of the pH of the forward-extraction buffer. Back extraction conditions were as in Figure 2. Error bars signify a 95% confidence level.
In an attempt to correlate the results from the two surfactant systems, we looked at the water content as a measure of the size of the micelles. In general and as expected, the water contents of the CTAB reversed micelles were much larger than those from the TOMAC reversed micelles. Typical values ranged from approximately 10 in the TOMAC system to 50 at 500 mM NaCl and 68 at 300 mM NaCl in the CTAB system. These numbers parallel those of Krei and Hustedt (1992), who reported maximal water content of CTAB reversed micelles to vary from -115 at 100 mM NaCl to -60 at 300 mM NaCl and -50 at 500 mM NaCl. The water content does not vary greatly with the pH of the initial phase, but salt concentration does have a great effect. A single correlation between partitioning and micelle size using the two systems was not possible as the mechanism for reducing the water content in the CTAB system was increasing salt content, which affects not just micelle size but also the electrostatic interaction between the protein and the reversed micelle. Therefore, even though it stands to reason that the large micelle size is the reason for the high recovery in the CTAB system, it is not the only factor in determining protein solubilization.

In a test to determine the ability of reversed micelles to extract charged fusions from multicomponent systems, the control enzyme GA’CD0 was partitioned from clarified fermentation broth (adjusted to pH 7.2) into CTAB reversed micelles. This resulted in a recovery of 62%, as compared to approximately 70% from pure samples. Evidently the presence of broth constituents does not interfere with extraction in this system, so reversed micellar extraction may prove to be useful for recovery from fermentation broth.

Conclusions

Charged fusions can be utilized successfully as an aid to reversed micellar extraction under some conditions. In surfactant systems or under conditions in which small reversed micelles are formed, charged fusions can play a large role in increasing the amount of protein partitioned into the organic phase. The charged fusions do this by increasing the amount of
charge present on the protein for interaction with the charged surfactant head groups. However, in cases where relatively large reversed micelles are already formed, the presence of charged fusions did not increase performance. It appeared that the charge required for partitioning was sufficient for the control enzyme and that added charges were unnecessary. Only in situations where the overall charge was very low or micelle size was small, \textit{i.e.}, at pH's near the isoelectric point or at high salt concentrations, were there even slight differences in the behavior of the mutants.

It is worthwhile to note the drastic differences in the two extraction systems. The CTAB system enjoyed great superiority in the amount of protein recovered over the TOMAC system, though it suffered from extreme variability that was not paralleled with TOMAC. Finally, it is worth mentioning that in this study we have looked only at recovered, active protein. While this is, in an overall view, a more important criterion for evaluation than just the amount partitioned into the reversed micellar phase, it may also cloud the role of protein charge in the extraction process. Benefits from the charged fusion tail in forward extraction may be offset by hindering back extraction, so partition coefficients may, in actuality, be higher than reported.

\textbf{Acknowledgment}

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Literature Cited


USE OF ION EXCHANGE MEMBRANES FOR SELECTIVE RECOVERY OF ASPERGILLUS AwAMORI GLUCOAMYLASE AND PHAGE T4 LYSOZYME

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ABSTRACT

Our research examined whether enhanced separation and purification of mutant enzymes could be obtained on ion-exchange membranes. Solutions of three mutants of Aspergillus awamori glucoamylase were passed through an anionic exchange membrane, as well as one mutant of T4 lysozyme through a cationic exchange membrane. The mutant enzymes were modified by adding "charged fusions", polypeptides of either aspartic acid residues to increase the overall negative charge of the enzyme or arginine residues to increase the overall positive charge. The effect of the mutations on the purification of glucoamylase from a "modified" fermentation broth were examined at two different elution pH's, 4.5 and 6.0.

The use of the charged fusions provided significantly improved purification capabilities over control versions. Both the small scale glucoamylase runs and the scaled up experiments had overall purification factors of around two, with a peak purification factor of near 7 for GA'CD10. Elution of glucoamylase at pH 4.5 did not lead to an increase in separability as compared to that obtained at pH 6.0.

Initial trials using a purified lysozyme mutant showed significant binding capabilities. Further experiments with this protein need to be done to determine scale up potential.

INDEX DESCRIPTORS: fusion proteins, purification fusions, protein purification, ion exchange membrane
The biochemical processing industry has in recent years made great strides in techniques for producing genetically engineered proteins; however, methods for separating and purifying these proteins cheaply have not shown similar growth. Many purification techniques that have proved successful for small quantities in the research laboratory are simply too difficult and costly to scale up.

One approach to this problem that has been taken in our lab with favorable results is the purification of genetically altered proteins using ion exchange membranes. An ion exchange membrane has a matrix of charged groups on it which attract oppositely charged proteins as they are passed through the membrane. Figure 1 is a schematic of an anionic exchange membrane, which in this case has positively charged quaternary amine groups which bind to negatively charged proteins. An advantage to using an ion exchange membrane is that binding and elution cycles can be run much more quickly than conventional ion exchange chromatography. In addition, the selectivity of this technique may be enhanced by using mutant enzymes containing additional charged amino acids in the form of “purification fusions”; these extra amino acids create a region of high charge density on the protein that increases the protein’s ability to bind to ion exchange groups. This method has worked well for purification of the enzyme β-galactosidase using both hollow fiber membranes (Heng et al., 1992) and ion-exchange membranes (Thiem and Heng, 1993). Our work used two different enzymes, glucoamylase from *Aspergillus awamori* and T4 lysozyme, to continue investigation of ion-exchange membranes as a method for separation and purification of charged fusion proteins.

**MATERIALS AND METHODS**

**Microporous Membranes**

The membrane used for small scale experiments with glucoamylase was a 25 mm O.D. QUAT Acti-Disk™ Separation and Purification Cartridge (FMC BioProducts).
Figure 1. Schematic diagram of a microporous ion-exchange membrane.
The large scale glucoamylase and lysozyme experiments utilized the 50 mm O.D. QUAT and CM Acti-Disk™ cartridges, respectively. The QUAT cartridge has positively charged quaternary amine groups while the CM cartridge has negatively charged carboxymethyl groups.

Fermentation and Preparation of Glucoamylase

The gene for glucoamylase (a gift from Cetus Corporation, Emeryville, CA) was genetically altered to produce mutants with additional charged residues in the form of a "fusion tail" on the carboxyl terminus of the protein (Suominen et al., 1993). Table 1 shows the amino acid sequences of the tails of the three mutants used in our experiments.

The microorganism used for production of the mutant glucoamylases was the yeast *Saccharomyces cerevisiae*. Cells were grown in yeast SD minimal media supplemented with 100 mg/l l-histidine using 2% glucose as a carbon source. Fermentations were performed at the ISU Fermentation Facility at 30°C and pH 4.5, with cells being harvested at 5–7 days (Forney and Glatz, 1993). In this expression system, glucoamylase is secreted, so the cells were removed by using a hollow fiber microfiltration apparatus with a 0.1 µm nominal pore size (Amicon, Danvers, MA). The broth permeate was concentrated from 50 liters to 500 ml using a spiral wound hollow fiber apparatus with a 10 kD molecular weight cutoff (Amicon, Danvers, MA) and then diafiltered with 5 volumes of water, at which point it was stored at 4°C. Before final use the stored material was centrifuged at 10,000 rpm and 4°C for 10 minutes and the supernatant passed through a 0.1 µm filter.

Table 1: Amino acid sequences for purification fusions

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>GA'CD0</td>
<td>...Gly Ser Met Ala Tyr</td>
</tr>
<tr>
<td>GA'CD5</td>
<td>...Gly Ser Met Ala (Asp)5 Tyr</td>
</tr>
<tr>
<td>GA'CD10</td>
<td>...Gly Ser Met Ala (Asp)10 Tyr</td>
</tr>
</tbody>
</table>
Fermentation and Preparation of Lysozyme

The gene for the triple mutant lysozyme was a gift from Brian Matthews (University of Oregon). The triple mutant has glutamic acids substituted for lysines at amino acid residues 16, 135 and 147 (Dao-Pin et al., 1991). A purification fusion consisting of the residues Arg-Val-(Arg-Val)_4-Arg was added to this mutant to create the U3V mutant, which was used in these experiments (Bakir, U., Iowa State University, personal communication, 1993).

The microorganism used for production of lysozyme is *Escherichia coli*. Cells were grown in Luria Bertani media, supplemented with 100 mg/l ampicillin, at 32°C in an Environ-Shaker 3597 (Lab-Line, Melrose Park, IL). Growth proceeded for about four hours, at which point IPTG (isopropyl β-D-thiogalactopyranoside) was added to induce lysozyme production. After 90 minutes, the cells were harvested by centrifugation. The cell pellets were then resuspended in 20 mM sodium phosphate buffer, pH 6.5, and sonicated to disrupt the cells, with the cell debris removed by centrifugation. The supernatant was dialyzed into 20 mM Tris, pH 7.25, then purified on an CM-Sephadex ion exchange column (Sigma Chemical, St. Louis MO) using a linear 0–300 mM sodium chloride gradient. Fractions were tested for activity, with the purified lysozyme fraction dialyzed into 20 mM Tris solution, pH 7.25, and stored at 4°C for use with the ion exchange membrane experiments.

Feed Preparation

The preparations of each of the three mutants of glucoamylase were diluted 1:1 with 50 mM sodium acetate and then adjusted to pH 6.0. The protein concentration for the set of experiments at elution pH 4.5 was approximately 500 µg/ml, while the protein concentration for elution pH 6.0 varied between 400–800 µg/ml. The feed for the large scale studies was adjusted to pH 6.0, and no further adjustments were made to either those solutions nor the lysozyme solution.
Membrane Loading and Elution of Glucoamylase

For those samples eluted at pH 4.5, the membrane was equilibrated with about 20 ml of 50 mM sodium acetate buffer, pH 6.0. Five milliliters of the feed solution were passed through the membrane six times using a syringe. The membrane was then washed with six 5 ml passes of equilibration buffer to remove any loosely bound protein. The removal of the bound protein was done by step gradient elution using 2 ml each of 50 mM sodium acetate solutions, pH 4.5 with NaCl concentrations varying from 0.1 M -0.5 M in 50 mM graduations. One and a half milliliter samples of the feed, effluent, buffer wash, and each of the salt steps were set aside for protein and activity assays. Samples were stored at 4°C until assaying.

For the samples eluted at pH 6.0, the membrane was again equilibrated with 50 mM sodium acetate buffer pH 6.0, though the solution was passed through the membrane using a Tris pump (ISCO, Lincoln, NE) at a flow rate of 5 ml/min. for ten minutes. The feed was then passed through the membrane at 1 ml/min. and recycled twice. After loading, the membrane was washed with 20 ml of equilibration buffer to remove loosely bound protein. The protein was removed with a step elution from 0.1 to 0.5 M NaCl in 50 mM sodium acetate at pH 6.0, with a step size of 100 mM NaCl. A solution of 50 mM sodium acetate with 1 M NaCl was also used to ensure complete protein removal. Elution buffers were passed through the membrane in 3 ml aliquots. Syringes were attached to either end of the cartridge and each salt solution was passed through four times. Samples of the feed solution and all effluents were saved for protein and activity assays.

For large scale operation, the membrane was equilibrated with 50 mM sodium acetate at pH 6.0 and then loaded with glucoamylase feed solution. The loosely bound protein was removed with 50 mM sodium acetate and 100 mM sodium chloride, pH 6.0. Elution of the bound protein was done in one step using a 50 mM sodium acetate buffer with 0.5 M sodium...
chloride, pH 4.5. All steps were done using a peristaltic pump at a flowrate of 1 ml/min. The duration of each step in the process was determined by the protein concentration of the effluent as indicated by absorbance at 280 nm on a Model UA5 detector (ISCO, Lincoln NE).

Membrane Loading and Eluting of Lysozyme

The cation exchange cartridge was equilibrated with 20 mM Tris pH 7.25, and 1 ml of sample was loaded. The membrane was then washed with 20 ml of the equilibration buffer. Removal of bound protein was done by step elution, with 1 ml each of the same nine salt concentrations as for the small scale application of glucoamylase in 20 mM Tris pH 7.25.

Membrane Care

The membranes were regenerated with 20 ml of either 1 M or 2 M sodium chloride. They were then rinsed with 20 ml of deionized water, 20 ml of absolute methanol, and 20 ml air to force out any remaining methanol. Cartridges were stored at room temperature until the next use.

In large scale use, the membranes were regenerated with 2 M sodium chloride at pH 3.6. Cartridges were rinsed with about 50 ml each of deionized water and methanol, dried with air, and stored at room temperature.

Assays

Activity and protein assays were performed on each sample to determine its relative purity.

Activity assays of glucoamylase were performed using a modified procedure from Svensson et al. (1982). One activity unit is defined as the amount of glucoamylase required to release one micromole of glucose from soluble starch in one minute. One hundred microliters of sample was added to 2 ml of a two percent soluble starch solution in 50 mM sodium acetate, pH 4.5. Three hundred microliter aliquots were drawn off at desired intervals, and
the reaction was stopped by pipeting the aliquots into tubes containing 200 μl of 2.5M Tris/HCl, pH 7.0. Glucose concentration was determined using the method of Banks and Greenwood (1971) using a Tris/HCl/glycerol buffer.

The lysozyme activity assay was performed by adding samples to a Micrococcus lysodeikticus cell suspension, then measuring the percent transmittance at 540 nm as a function of time (Parry et al., 1965). The activity was calculated from the slope of this line.

The protein assay used for both enzymes was the Pierce BCA Protein Assay (Pierce, Rockford, IL), both standard and enhanced protocols, with BSA as a standard.

**DISCUSSION OF RESULTS**

The purity of a sample is indicated by its specific activity, which is defined by the amount of activity per unit mass of protein in the sample. The specific activity is calculated by dividing the activity in U/ml by the protein concentration in mg/ml, both determined by the assays described earlier. A purification factor was also calculated for each sample, which is a comparison of a sample’s purity compared to that of the initial feed. It is a dimensionless quantity calculated by dividing the specific activity of a sample by the specific activity of the feed, and indicates the relative number of times the protein was purified.

**Small Scale Glucoamylase Experiments**

The results of the small scale glucoamylase runs with elution pH 6.0 are shown in Figures 2 and 3 and Table 2. Figure 2 shows the percentage of protein and activity recovered at each salt elution step, as well as the specific activity. The percentage of protein recovered is defined here as the amount of protein in a given fraction divided by the total amount of protein recovered from the membrane. Note that the protein elution peak for each mutant is at the 0.2 M elution step. Also note here that the activity peak shifts from between 0.2–0.3 M for
Figure 2a. Activity (■) and protein (●) profiles for GA'CD0. Specific activity (△) is calculated as the ratio of glucoamylase activity (U) to protein content (mg). Loading conditions were 25 mM sodium acetate, pH 6.0. Elution conditions were 50 mM sodium acetate, pH 6.0 with NaCl step gradient.
Figure 2b. Activity (■) and protein(●) profiles for GA'CD5. Specific activity (∆) is calculated as the ratio of glucoamylase activity (U) to protein content (mg). Loading conditions were 25 mM sodium acetate, pH 6.0. Elution conditions were 50 mM sodium acetate, pH 6.0 with NaCl step gradient.
Figure 2c. Activity (■) and protein (○) profiles for GA’CD10. Specific activity (△) is calculated as the ratio of glucoamylase activity (U) to protein content (mg). Loading conditions were 25 mM sodium acetate, pH 6.0. Elution conditions were 50 mM sodium acetate, pH 6.0 with NaCl step gradient.
Figure 3. Purification profiles of step gradient elution of GA'CD0, GA'CD5, and GA'CD10.
Table 2: Results for mutant glucoamylase trials at elution pH=6.0.

<table>
<thead>
<tr>
<th></th>
<th>GA'CD0</th>
<th>GA'CD5</th>
<th>GA'CD10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed Protein (mg)</td>
<td>19.4</td>
<td>10.6</td>
<td>18.0</td>
</tr>
<tr>
<td>Feed Activity (U)</td>
<td>31.6</td>
<td>52.2</td>
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<td>Feed Specific Activity (U/mg)</td>
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<tr>
<td>% Feed Activity Captured</td>
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<td>93.1</td>
<td>76.1</td>
</tr>
<tr>
<td>% Captured Activity Recovered</td>
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<td>85.5</td>
<td>87.7</td>
</tr>
<tr>
<td>Overall Purification Factor</td>
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</tr>
<tr>
<td>Peak Specific Activity (Ionic Strength)</td>
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<td>19.0 (0.3 M)</td>
<td>19.9 (0.5 M)</td>
</tr>
<tr>
<td>Peak Purification Factor (Ionic Strength)</td>
<td>4.5</td>
<td>3.9 (0.3 M)</td>
<td>7.2 (0.5 M)</td>
</tr>
</tbody>
</table>

GA'CD0 to 0.3 M for GA'CD5 to between 0.3–0.4 M for GA'CD10. The specific activities of glucoamylase generally increase with increasing tail length. Additionally, the elution steps at which the maximum specific activity occurs also increases with tail length, from 0.3 M for GA'CD0 to 0.5 M for GA'CD10, with GA'CD5 in the middle at 0.3–0.4 M. All of these trends point to enhanced binding of the proteins with purification fusions to the ion exchange
membranes relative to the control version. The purification factors for each of the salt elution steps are shown in Figure 3. This number generally increased both with the increasing ionic strength of the salt elution steps and with increasing tail length. Finally, Table 2 gives results for representative experiments.

Shifting the elution pH from 6.0 to 4.5 resulted in a marked decrease in the ionic strength required to elute the proteins and also a decrease in the specific activities in the fractions. This result is shown in Figure 4 where the activity elution peak is at the 0.15 M elution step instead of the 0.3 M step as shown in Figure 2. This is due to a decrease in the net charge on the protein (estimated net charges for glucoamylase are given in Table 3) and thus smaller interaction with the ion exchange groups. Since more of the target protein was released at lower ionic strengths as are the undesired proteins, the resultant purity was lower than seen at pH 6.0.

**Large Scale Glucoamylase Experiments**

A large scale glucoamylase procedure has been examined for the GA'CD5 protein. The feed effluent, the 0.1 M elution step, and the 0.5 M elution step were all saved. The results of a number of purification trials with the GA'CD5 mutant protein are shown in Table 4. In all cases, the purification factor is near two with the resulting specific activity being equal to that of affinity chromatography purified GA'CD5 (Forney, C. unpublished results). Further experiments are under way to recycle the feed effluent and the 0.1 M elution step by reloading those fractions on the ion exchange membranes.

**Lysozyme Experiments**

Figure 5 shows the specific activities obtained at each step in the elution of lysozyme from a cation exchange membrane; the peak at 0.3 M indicates that significant binding is being obtained by this method. More experiments are needed to verify this behavior over an expanded pH range.
Figure 4. Step gradient elution of activity at an elution pH of 4.5. Loading conditions were 25 mM sodium acetate, pH 6.0. Elution conditions were 50 mM sodium acetate, pH 4.5 with NaCl step gradient.
Table 3: Estimated net charge on glucoamylase at pH 4.0–4.5, 6.0. Charge estimations determined using Henderson-Hasselbalch equation with amino acid pK's from Stryer (1988).

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Table 4: Large scale glucoamylase results for GA'CD5

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<th>0.5 M Elution Sample Spec. Act. (U/mg)</th>
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Step gradient elution of lysozyme activity. Loading conditions were 20 mM Tris, pH 7.25. Elution conditions were the same buffer with NaCl step gradient.
CONCLUSIONS

In the small scale application of ion exchange membranes for glucoamylase, the results show that excellent purification can be achieved. Specific activities are similar or superior to those obtained using affinity chromatographic techniques. We have also shown that the purification fusions enhance binding of the target protein to the membrane relative to the control versions.

The large scale application of the ion exchange membranes seems to work very well, and the technique can be used efficiently for separation and purification of large quantities of glucoamylase with a significant decrease in processing time over affinity chromatography. More experimentation is needed to determine whether using the longer tailed mutants enhances separation on a large scale and the effect of charge on separability of lysozyme.

ACKNOWLEDGMENTS

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REFERENCES


GENERAL CONCLUSIONS

Reversed micellar extraction, even coupled with charged fusion proteins, suffers from the same problems that plague other purification methods, i.e. the trade-off between high recovery and high selectivity. In the case of reversed micellar extraction, the trade-off was epitomized by the TOMAC and CTAB extraction systems.

Charged fusions play a discernible role when used in conjunction with the TOMAC surfactant system. With this surfactant, there were consistent differences in the behavior of the mutants, with the presence of the tail providing for more effective extraction. Because of this, one might expect that selectivity would be high in this system. However, recoveries using TOMAC were generally quite low (maximal recovery less than 40%).

The CTAB surfactant system proved to have much higher maximal recoveries than the TOMAC system, but it suffered from its inability to discern between the mutants, even under stringent conditions of very low pH and/or high salt. This lack of selectivity was most apparent at pH values near the pI's of the various mutants, where charge differences at a given pH were largest on a percentage basis. Even here yields of the mutants were indistinguishable, with partitioning seemingly dependent more on the pH of the solution than the charge on the protein. Additionally, the CTAB system proved to be much more susceptible to day to day variability than the TOMAC system, for which recoveries were consistent over a period of years.

The use of ion-exchange membranes as a step in glucoamylase purification provided selective recovery of the protein with specific activities for GA'CD5 similar to or superior to that obtained using affinity chromatography techniques but with a reduction in processing time of greater than 75%. Though used in this project as a preparatory step for further experimentation, its combination of high throughput and short turn-around time make this method a clear winner over extraction at this stage.
Potential directions for future work lay in attempting to solve the quandary of high selectivity or high recovery. Only two cationic surfactant systems were studied here, the two having very different properties. Krei and Hustedt (1992) used a larger number of surfactants in their research and found TOMAC and CTAB to be on the opposite ends of the spectrum with regard to water number. Perhaps using a surfactant intermediate in its properties, such as benzyl dodecyl bis(hydroxyethyl) ammonium chloride (BDBAC), might allow selective extraction with high recoveries. Many other factors were not considered, such as the organic solvent, the temperature of the extraction (Dekker et al. (1991) performed forward extraction at 4°C and found increased solubilization over that at 20°C), various types of cosurfactants, and the like.

On the protein side, a different enzyme could be experimented with. Glucoamylase is on the large side for reversed micellar extraction, and a smaller enzyme may prove to be very useful for investigating the charge-dependent nature of the extraction. Modifications similar to those performed on Phage T4 lysozyme (Dao-Pin et al., 1991) combined with charged fusions would give the ability to vary charge on a protein in both a distributed and concentrated fashion, and the differences in behavior would be very interesting from a charge asymmetry standpoint.
LITERATURE CITED


APPENDIX: PROTEIN NET CHARGE ESTIMATION

Estimated net charges for the glucoamylase charged fusions were calculated (J. R. Luther, personal communication) using the Henderson-Hasselbalch equation, given below. The residues that were used in this calculation were determined by examining the three dimensional structure of glucoamylase to determine which amino acids were accessible to the solvent and thus ionizable (Luther, 1994).

The Henderson-Hasselbalch equation is

\[ \text{pH} = pK + \log \frac{[R^-]}{[RH]} \]  

for acidic groups, with a similar expression for basic groups. It can be rearranged to give the fraction of ionizable groups that are dissociated for each type of amino acid, and the net charge on the protein, \( Z_p \), can be determined by summing over all of the ionizable amino acids, \( i.e. \)

\[ Z_p = \sum_{i} \frac{n_i}{1 + 10^{(pH - pK_i)}} - \sum_{j} \frac{n_j}{1 + 10^{(pK_j - pH)}} \]  

where \( n \) is the number of residues of type \( i \) (basic groups) or type \( j \) (acidic groups) (Luther, 1994). For the purpose of this estimation, only a single value for the pK of each amino acid was used. These values (combined from published lists by Lehninger (acidic pK's) and Tanford (basic pK's) (Luther, 1994)), as well as the number used for each of the mutants is given in Table A1. Table A2 gives the estimated net charge of the glucoamylase mutants GA'CD0, GA'CD5, and GA'CD10 as a function of pH over the range used in papers 1 and 2, while Figure A1 shows the titration curve for each of the mutants.

LITERATURE CITED

### Table A1.
Dissociation constants (Luther, 1994) and number of charged residues used in charge estimation per mutant.

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Table A2. Estimated net charges for mutants GA'CDO, GA'CD5, and GA'CD10 as a function of pH.

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Figure A1. Predicted titration curves for GA'CD0, GA'CD5, and GA'CD10. Calculations made using pK's and charged residue numbers from Table A1.