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The extraction of Cytochrome C and DsRed2 into reverse micelles

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THE EXTRACTION OF CYTOCHROME C AND DSRED2 INTO REVERSE MICELLES

by
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Dr. Stephanie Farrell

ABSTRACT

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THE EXTRACTION OF CYTOCHROME C AND DSRED2 INTO REVERSE MICELLES
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Cytochrome c and DsRed2 were successfully extracted into reverse micelles by the contacting of an aqueous protein-containing phase with an organic phase. Two important properties that differentiate the extraction profiles of these proteins are pI and size. Cytochrome c is a relatively small, monomeric protein with a pI of 10.6. It was easily extracted into reverse micelles with the anionic surfactant AOT. DsRed2, however, is a large tetramer with a pI of 6.3. It could not be extracted into AOT, but was extracted with the cationic surfactant CTAB, and with a wider error range than cytochrome c. CD data indicate the secondary structure of the proteins may change with solubilization into reverse micelles, despite absorption interference from the micelles. The results of this thesis suggest that extraction of certain proteins into reverse micelles is a viable primary separation step for the recombinant biotechnology industry. However, each process will have to be optimized to the protein of interest, as protein extraction is specific to certain properties and is extremely sensitive.

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CHAPTER I

Introduction

Background

The solubilization of hydrophilic proteins in reverse micelles (RMs) has great potential as a primary separation step in the downstream processing of recombinant biotechnology. In the biotech industry, downstream processing is expensive; it is responsible for the majority of production costs. Downstream processing is also time-consuming and complex. The proteins of interest are similar to other components in the growth media and must retain their biological activity. The process must achieve a high separation with a large throughput. Therefore, any new technology that reduces the cost, the amount of energy needed, or the complexity will be extremely beneficial and important to the process.[1-3]

Recombinant processes are very specific to the application, to the source of the biomolecule (mammalian, microbial, etc.) and to the properties of the protein. Proteins produced from recombinant bacteria will need to be separated from the fermentation broth. First, the bacterial cells are harvested by centrifugation or by membrane filtration. The cells may be washed by diafiltration to remove soluble impurities; this can also set the pH and the salt concentration. In order to break the cell wall and release the proteins, the cells are subjected to chemical treatments such as ethylenediaminetetraacetic acid

(EDTA) and lysozyme before mechanical disruption. The resulting cell lysate is then clarified by centrifugation or by membrane filtration. If the protein present is an inclusion body, it is located in the cell pellet, but if soluble, the supernatant. The inclusion bodies are washed, denatured, and allowed to refold. After primary recovery and separation, the protein is then purified, usually by several filtration (ultra, nano, tangential flow) and chromatography (expanded bed adsorption, ion exchange, hydrophobic interaction, size exclusion, affinity) steps. A final purification step may include sterile filtration to remove bacteria or membrane filtration to remove viruses. Proteins recombinantly grown in mammalian cells are produced extracellularly and the process therefore avoids cell lysis.[4] There are several reviews available detailing the current and potential bioseparation equipment.[5-8]

Liquid-liquid extraction of proteins by reverse micelles can be scaled up to operate continuously as a primary separation step with readily available liquid extraction equipment such as mixer-settlers and contactors.[9] In fact, RMs have been shown to lyse whole bacterial cells, with a back extraction step recovering the protein of interest for purification, greatly reducing the pre-chromatography steps.[10] The characteristics of RMs and the factors and forces influential in protein solubilization are detailed below.

Protein Solubilization in Reverse Micelles

In reverse micelles, the polar head groups of the surfactant are ordered around a water pool, while the hydrophobic tails of the surfactant interact with the bulk organic solvent. Figure 1 below depicts a cartoon of protein solubilization in RMs. A variety of surfactants exist, with the anionic AOT (sodium

diethylhexylsulfosuccinate)/isooctane/water ternary system the most well studied since it does not require a cosurfactant.

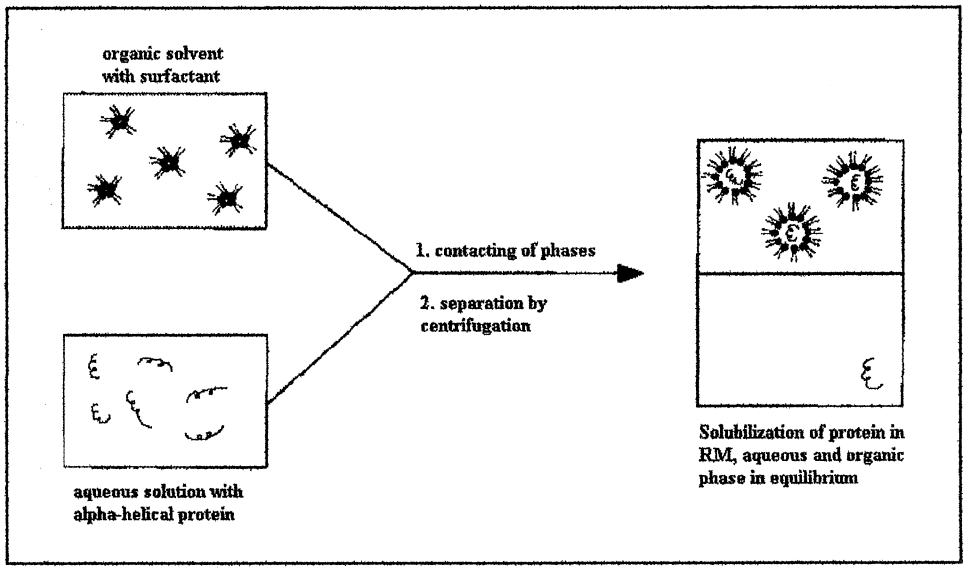

Figure 1. Cartoon Representation of Protein Solubilization in RM, adapted from Melo et al.[11]

Micellar collisions allow the water pools to occasionally exchange contents regardless of the chemical properties of the contents. The size of the micelle depends on the ratio of the concentration of water to the concentration of surfactant (in moles), known as W_0, provided the surfactant concentration is greater than the critical micelle concentration (CMC). The parameter, W_0, is also considered important for protein selectivity and enzyme activity. At low values of W_0, all water molecules in the RM are bound to the surfactant head groups, causing the micelles to be rather inflexible. As W_0 increases ($W_0 > 10$), some water molecules are attached to the surfactant head groups and some are free in the core of the RM, giving the micelles a dynamic flexibility. Also, as W_0 increases, the size of the water pool increases.[11]

The water pool has different physical and chemical properties than bulk water and is dependent on the conditions of the micelle. Therefore, it is necessary to experimentally analyze the conditions required for protein solubilization and characterize the properties of the micelle. Protein solubilization in RMs is driven by both electrostatic and hydrophobic interactions. These driving forces are affected by many properties. In the aqueous phase, the protein concentration, the salt type and concentration, the buffer type and concentration, and the pH affect the driving forces. In the organic phase, the surfactant type and concentration, the solvent type and concentration, and the presence of co-surfactants or co-solvents affect the driving forces. With respect to the micelle, the size and shape of the micelle (characterized by W_0) affect the driving forces. The size and shape, isoelectric point (pI), charge distribution, and hydrophobicity of the protein influence the location of the protein in the RM (water pool, head or tail of surfactant) and also the driving forces. A variety of biophysical chemistry techniques has been used to characterize the micelles, such as dynamic light scattering, fluorescence, circular dichroism (CD), Förster resonance energy transfer (FRET), and nuclear magnetic resonance (NMR).[12-19] There are several excellent reviews on the important factors in extracting proteins into reverse micelles.[20-24]

Forward Transfer Methods

There are three methods for transferring a protein into a RM. The phase transfer method, the most applicable to continuous processing in the biotech industry, involves contacting an aqueous phase containing the protein and an organic phase. The resultant reverse micelles in the organic phase are in equilibrium with the bulk aqueous phase. The injection method involves injecting a solution with soluble protein into an organic phase

with empty RMs. The solid-liquid method involves mixing lyophilized protein with an organic phase containing empty RMs. Hashimoto et al.[25] added solid, denatured ribonuclease A to empty micelles and allowed the enzyme to refold. The study was applicable to proteins that are produced as inclusion bodies, which may need to be separated and refolded.

After solubilization, the protein may be located in the water pool, in contact with the polar head group, or in contact with the hydrophobic tail. The location depends on the protein and its properties.

Electrostatic Forces

Electrostatic forces in this context refer to the interactions between the charges on the polar head of the surfactant and the charges on the protein. They are usually strongest during forward transfer when a protein is below its pI and is extracted into a RM with an anionic surfactant or when a protein is above its pI and is extracted into a RM with a cationic surfactant. The presence of salt or buffer can interfere with electrostatic forces, either by the "salting out" effect or by Debye screening due to a reduction in the Debye length. Examples of the influence of electrostatic interactions are presented below.

pH

If electrostatic interactions are the main driving force for protein solubilization, then the pH of the aqueous phase and water pool are important. Wolbert et al.[26] studied the effect of aqueous pH on the solubilization of nineteen proteins into trioctylmethylammonium chloride (TOMAC)/nonylphenol pentaethoxylate (Rewopal HV5)/isooctane and AOT/isooctane. With few exceptions, the researchers were able to extract the proteins that were oppositely charged of the surfactant, confirming the

importance of electrostatic forces for forward transfer. As stated previously, a protein in an environment with the pH lower than its pI will be able to transfer with an anionic surfactant, and vice versa. Wolbert et al. determine that the size of the protein is also important in determining the optimal pH for transfer, as a larger protein needs a larger driving force, i.e. pI – pH. They also discovered that at higher ionic strengths, a larger difference is needed for pI – pH.

Salt Concentration

A low salt concentration is needed for the forward transfer of proteins to prevent emulsions. For extraction that is driven mainly by electrostatic interactions, the salt concentration becomes an important parameter in solubilization, along with pH. At higher ionic strengths, the solubilization of a protein decreases as the amount of ions increases. The presence of ions decreases the electrostatic interactions between the head group of the surfactant and the protein by Debye screening.

Göklen and Hatton[27] solubilized positively-charged cytochrome c into the anionic AOT/isooctane system rapidly at low ionic strength and then back transferred the protein at high ionic strength, but more slowly. Forward transfer was relatively fast (order of seconds) while back transfer was slower (order of minutes). These results indicated to the researchers that the extraction of cytochrome c is dominated by electrostatic interactions between the charged protein and the inside of the micelle. Göklen and Hatton suggested that the type of salt may influence the driving force, as a protein may interact differently with different salts.

Surfactant: Type and Concentration

The type of surfactant (anionic, cationic, nonionic[28], zwitterionic[29]) needed is dependent on the properties of the protein. Some surfactants need a co-surfactant or co-solvent to form stable reverse micelles. New surfactants are also being designed for better protein transfer.[30] AOT (anionic) and CTAB (cetyl trimethylammonium bromide, cationic) are the most commonly studied surfactants. Melo et al.[31] discovered from dynamic light scattering that 1-hexanol increased the size of the cutinase-encapsulated AOT micelle. The change in size of the micelle changed the interfacial characteristics, which then prevented protein denaturation.

The surfactant concentration required for transfer is related to the concentration of protein in the aqueous phase. Larger concentrations of protein require larger concentrations of surfactant. However, too much surfactant can lead to crowding and difficulty in back transfer. Ichikawa et al.[32] investigated the extraction of cytochrome c into AOT/isooctane with respect to the amount of solubilizing water in the RM. The researchers determined the minimal AOT concentration in different conditions, defined as the AOT concentration for 100% extraction. They found that it increased as the aqueous protein concentration increased and as the ionic strength increased, and was affected by the pH in the aqueous phase.

Hydrophobic Forces

Hydrophobic forces refer to interactions between the hydrophobic regions on the protein and the hydrophobic tails of the surfactant. They are usually responsible for back transfer and are strongly dependent on temperature.

Pires and Cabral[33] extracted cytochrome b5 into CTAB/cyclohexane/decanol. By adjusting the parameters, the researchers were able to extract the protein with either electrostatic or hydrophobic interactions as the main driving force. Electrostatic forces extracted the protein at low ionic strength and a pH far from the pI. However, hydrophobic forces extracted the protein at a pH close to the pI. Hydrophobic forces were further confirmed for the latter case because the extraction was independent of ionic strength (above 0.4 M KCl) and the extraction was temperature-dependent. Hydrophobic forces have more influence at higher temperatures.

Hebbar et al.[34] investigated the solubilization of a large protein (66 kDa) into a nonionic surfactant, polyoxyethylene p-t-octylphenol (Triton-X-100)/toluene, and AOT/toluene. Bovine serum albumin (BSA) was not extracted into Triton-X-100/toluene reverse micelles even though the micelle size was larger than the protein. The researchers concluded there was not enough of a driving force between BSA and the nonionic surfactant. The protein was extracted into AOT/toluene at a pH higher than the pI, indicating the extraction was dominated by hydrophobic forces. Adding the protein to a mixture of Triton-X-100 and AOT in isooctane lowered the extraction efficiency, as the hydrophobic driving force decreased without a comparable increase in electrostatic interactions.

Backward Transfer

The traditional method of protein back-extraction from the organic phase to a new aqueous phase involves increasing the ionic strength and changing the pH to cause unfavorable electrostatic interactions between the surfactant and the protein and to expel the protein by size exclusion due to the added salt presence.

Alcohol addition to RMs[35] has been known to help back-extraction by controlling micellar interactions. Other back-extraction techniques involve the use of silica[36] or the addition of a counter-ionic surfactant.[37]

Realistic Conditions: Mixtures and Broth

Once the requirements for solubilization of small proteins in RMs were established, this knowledge was applied to crude and artificial mixtures of proteins.[38,39] Bansal-Mutalik et al.[40] successfully extracted alkaline phosphatase from the periplasm of *E. coli* using several surfactants, after the cell culture had been centrifuged. Jarudilokkul et al.[41] selectively separated 3 proteins (cytochrome c, lysozyme, and ribonuclease A) from filtered fermentation broth using AOT/isooctane and discovered that low molecular weight positively charged amino acids and phospholipids in the broth acted as co-surfactants.

Scale-Up

Small scale-up of reversed micellar protein extraction has been done with a variety of equipment: membrane reactor,[42,43] 3-phase fiber reactor,[44] and high-speed counter-current chromatography column.[45] Stuckey et al.[46,47] chose a Graesser contactor for scale-up because the contactor prevented emulsions from forming, a common problem in reverse micelle scale-up.

Other Biomolecules

A brief literature search will also uncover other biomolecules that can be extracted: nucleic acids, enzymes, bacteria, whole cells, etc. The field of biocatalysis has added much insight to the extraction of proteins (enzymes) into reverse micelles. If the activity of an enzyme is not compromised by solubilization into a RM, the reaction can

occur in the RM after the addition of substrates. Biocatalysis in RMs is especially beneficial for enzymes with insoluble substrates.[48,49]

Proteins in This Thesis

The proteins used in this thesis are cytochrome c (horse heart) and DsRed2. These proteins are different in size, secondary and tertiary structure, and pI. The backgrounds of these two proteins are described below.

Cytochrome c

Cytochromes are hemeproteins that alternate between Fe^{2+} and Fe^{3+} oxidation states during electron transport. Cytochrome c is associated with the inner mitochondrial membrane, where it is involved in the electron transport chain. It is responsible for the transfer of electrons from cytochrome c reductase (Complex III) to cytochrome c oxidase (Complex IV) for the eventual production of adenosine triphosphate (ATP).[50] Also, the protein can disengage from the mitochondrion and activate apoptosis.[51]

The properties of cytochrome c are well known. In fact, it is used as a standard for electrophoresis and mass spectrometry, among other applications.[52] It is a single polypeptide chain with several α-helices.[53] Cytochrome c (horse heart) is well characterized in AOT reverse micelles. It is known to be located at the inner interface of reverse micelles.[54] Some properties of the protein are listed in Table 1 below.

DsRed2

The green fluorescent protein (GFP) cloned from the jellyfish *Aequorea victoria* has revolutionized the study of gene expression and of protein-protein interactions. Most GFP mutants have absorption wavelengths in the blue, green, or yellow wavelengths. However, fluorescent proteins with longer wavelengths are desirable for gene expression

and FRET. Matz et al., supported by Clontech Laboratories, hypothesized that GFP homologs might be found in nonbioluminescent organisms. The researchers were successful in cloning six fluorescent proteins from coral (Anthozoa species). The six proteins had only a 26-30% primary sequence identity with GFP, but shared a secondary structure, the β-can. One protein, from Discosoma species, was found to be significant due its reported red-shifted absorption maxima (558 nm) and emission maxima (583 nm).[55] Clontech commercialized the protein under the name DsRed, allowing the widespread research of its unique properties. DsRed2, with six mutations, has better solubility, brightness, and expression.[56]

Despite its potential, DsRed and its mutants have some disadvantages. The protein oligomerizes into a tetramer, which is problematic for many biochemical techniques.[57] It has been shown that the oligomerization is essential for the maturation of DsRed from its immature form to its mature red form.[58] Also, the protein has a long maturation time, 24 hours, according to Clontech.

There are very few research studies on the extraction of DsRed or DsRed2 into reverse micelles. Verkhusha et al.[59] determined the stability of GFP mutants (including DsRed) by subjecting the proteins to high pressure and AOT reversed micelles. The fluorescence of DsRed was found to be relatively stable solubilized in reversed micelles with W_0 from 10 to 30. Connell[60] solubilized DsRed2 in CTAB/isooctane/hexanol and determined the pH, salt, and surfactant extraction profiles. Properties of DsRed2 are listed in Table 1 below.

Table 1. Comparison of Proteins

Property	*DsRed2*	*Cytochrome c, from horse heart*
Color	hot pink	red
Molecular Weight (kDa)	103[61]	12[62]
Isoelectric Point (pI)	6.3[60]	10.6[50]
Excitation Maximum (nm)	563[63]	410[64]
Oligomerization	tetramer	monomer
Secondary Structure	β-can	α-helical
Extinction coefficient ($M^{-1}cm^{-1}$)	43,800[63]	106,000[65]

Far-UV Circular Dichroism

Circular dichroism (CD) is a very useful physical chemistry technique for studying the folding and binding of proteins and protein interactions. More importantly, it can help determine the structure of proteins. Near-UV (250-300nm) CD spectra can indicate characteristics of the tertiary structure. Far-UV (190-250nm) CD spectra can indicate the secondary structure. Proteins with α-helices have a positive band at 193nm and negative bands at 208 and 222nm. Proteins with β-strands have a positive band at 195nm and a negative band at 218nm. Extended (disordered) proteins have a negative band near 195nm and a low ellipticity above 210nm.[66]

CD measurements of proteins have been used in many studies to research the conformational changes between the protein in aqueous solution and in reverse micelles.[12,13,16,19] The usefulness of this technique for reverse micellar solutions is limited by the requirements of CD spectroscopy. For instance, many buffer systems are not compatible with CD spectroscopy, or must be used at extremely low concentrations.

Therefore, not all protein-containing reverse micellar solutions can be studied with identical conditions in CD. Also, reverse micelles can interfere with absorption in CD measurements.[18] Nevertheless, this technique can still provide important information about the structure of proteins in RMs.

CD measurements of cytochrome c have been studied under many conditions, including reverse micelles.[18] Naoe et al.[17] studied the CD data of cytochrome c (and lysozyme and ribonuclease A) to determine the influence of the protein and the micellar interface interactions on the structure of proteins in reverse micelles. The researchers concluded that the higher order content of cytochrome c in reverse micelles is strongly affected by micelle water content, micellar curvature, surfactant concentration, and solvent type due partly to the protein's location at the micellar interface. DsRed and DsRed2 have been studied with both near and far-UV CD,[67,73] but not under reverse micellar conditions. However, other spectroscopy techniques have been used on DsRed[59] or DsRed2[68] encapsulated in reverse micelles.

CHAPTER II

Purpose of Experiment

The aim of this thesis is to determine the extraction profiles of two proteins, cytochrome c and DsRed2, into reverse micelles. The surfactant concentration, salt concentration, and pH will be the factors studied to determine the influence of electrostatic or hydrophobic interactions on protein extraction. The well-studied surfactants, AOT and CTAB, will be employed. Also, the far-UV circular dichroic (CD) measurements will be analyzed to determine the secondary structure of the proteins in reverse micelles.

CHAPTER III

Materials and Methods

Cytochrome c

Cytochrome c from equine heart was purchased from Sigma (C2506).

Cytochrome c Surfactant Experiment

The aqueous phase consisted of 0.5g/L cytochrome c, 0.3M NaCl, and 0.02M Tris, pH ~9.7. The organic phase consisted of 5 to 50mM AOT (docusate sodium salt, Sigma, D4422) in isooctane (2,2,4-trimethylpentane, Sigma, 258776).

Cytochrome c Salt Experiment

The aqueous phase consisted of 0.5g/L cytochrome c, 0.02M Tris, pH ~9.7, and 0 to 0.8M NaCl. The organic phase consisted of 50mM AOT in isooctane.

Cytochrome c pH Experiment

The aqueous phase consisted of 0.5g/L cytochrome c, 0.3M NaCl, 50mM buffer, and pH from 4 to 11 (using acetic acid (Fisher, A491), sodium acetate (Fisher, BP333), sodium phosphate dibasic (Fisher, S373), sodium phosphate monobasic (Fisher, S381), sodium carbonate (Fisher, S495), sodium bicarbonate (Fisher, S233)). The organic phase consisted of 50mM AOT in isooctane.

Cytochrome c Analysis

900uL of both phases were contacted in a 2mL glass vial (Fisher, 03-339-22A) and were mixed for 1 hour on a rotisserie (Barnstead Labquake, 400110). The vials were then centrifuged for 20 minutes at 3000g. The top, organic phase was pulled off, placed in a micro-cell glass cuvette (Fisher, 14-385-914A, l = 1cm), and the absorbance was read at 410nm on a UV-visible spectrophotometer (Hewlett-Packard, 8453). The bottom, aqueous phase was not read due to surfactant contamination. The blank was isooctane since empty RMs in isooctane gave negative readings. The glass cuvettes were washed in a Vakuwash cell washer (Fisher, 14-385-937) with deionized water, isooctane, and acetone (Fisher, A949) between samples, and nitric acid (Fisher, A200) between runs and experiments.

Using the absorbance, the concentration of the protein extracted to the organic phase was determined from Beer's law:

$$A = \varepsilon * c * l,$$

where A is the absorbance at the excitation maxima (unitless), ε is the extinction coefficient in $M^{-1}cm^{-1}$, c is the concentration in M, and l is the pathlength of the glass cuvette in cm. The extraction yield was determined by

$$\%yield = \frac{\text{conc. of protein in organic phase}}{\text{actual initial conc. of protein in aqueous phase}} * 100$$

This equation removes error introduced by the literature values of ε.

Cytochrome c Far-UV CD

The circular dichroic measurements were taken with a Jasco J-810 CD spectropolarimeter in a 0.1cm round glass cuvette. The settings were as follows: standard

(100mdeg) sensitivity, 260 to 190nm, 1nm pitch, continuous scanning mode, 50 nm/min scanning speed, 16msec response, 5nm bandwidth, and 80-150 accumulation. The measurements of a blank aqueous solution (0.2M NaCl, 5mM phosphate buffer) were subtracted from the measurements of an aqueous solution of cytochrome c (30μM protein, 0.2M NaCl, 5mM phosphate buffer). The measurements of an empty micelle organic solution (resulting from 0.2M NaCl, 5mM phosphate buffer contacted with 50mM AOT in isooctane) were subtracted from the measurements of cytochrome c micelles (resulting from 30μM protein, 0.2M NaCl, 5mM phosphate buffer contacted with 50mM AOT in isooctane). Both sets of data were subjected to the means movement smoothing algorithm with 5-7 convolution width. The y-axis was converted from mdeg to molar ellipticity.

DsRed2 Growth and Purification

A frozen stock of *E. coli* BL21(DE3) cells inoculated with the plasmid pET21d, kindly provided by Sabrina Bédard in Joshua Wand's lab at the University of Pennsylvania, was grown in 10mL of DifcoTM LB Broth- Lennox (Fisher, DF0402170) and 10uL of 100mg/mL ampicillin (Fisher, BP1760) at 37°C and 120rpm in a VWR incubating orbital shaker. (The pET21d plasmid was previously subcloned from the Clontech pDsRed2 plasmid, which contained an ampicillin resistance gene and an IPTG-inducible DsRed2 fusion protein.) After three hours, the 10mL of culture was transferred to 200mL of broth and 200uL of ampicillin and allowed to grow overnight. The 200mL culture was then transferred to 1200mL of broth and 1.2mL of ampicillin such that the initial absorbance of the culture at 600nm was 0.1. The absorbance of the culture was monitored until $A_{600} \sim 0.8\text{-}1.0$, when 750uL of culture was set aside as a frozen stock

with 250uL of 60% glycerol (Fisher, BP229). Also, the culture was induced with 1.2mL of 100mg/mL IPTG (isopropyl-beta-D-thiogalactopyranoside, Acros, 302790010). The culture was then allowed to express for 4-5 days, until it was visibly hot pink. The culture was then centrifuged at 3000g for 15 minutes in a Forma-Scientific to harvest the cells. The supernatant was discarded and the cell pellet was resuspended in 15mL of 50mM Tris (Sigma 252859), 200mM NaCl (Sigma S9888), pH~8.0 and then placed in -70°C.

Next, the frozen cells were thawed and placed in a metal beaker. 25mM EDTA (ethylenediaminetetraacetic acid, Acros, 118432500) was added to the cells and allowed to stir for a half hour at room temperature. 100ug/mL of 22um-filtered chicken egg white lysozyme (Sigma, 117K0676) was then added to the cells and allowed to stir for an hour at room temperature. The cells were then subjected to five freeze-thaw cycles between -70°C and 30°C. The cells were then centrifuged for 3000g for 15 minutes. The supernatant was saved and diluted with 25mM Tris, 20mM NaCl, pH~8.5 until suitable for anion exchange purification.

5 to 25mL of dilute protein were anion exchanged on a Amersham Biosciences ÄKTA basic 10, starting at 25mM Tris, 20mM NaCl, pH~8.5 to 25mM Tris, 300mM NaCl, pH~8.5 in 6-7 column volumes. Examples of absorbance graphs for typical anion exchange chromatography for DsRed2 are in Appendix D.

The peak fractions were combined and centrifuged in an Amicon Ultra-15k (Millipore UFC905008) centrifugal filter device for 20 minutes at 3000g. The protein was then buffer-exchanged with 10mM Tris, pH~8.5 and centrifuged, repeating 4 times. The resulting concentrated protein was extracted from the filter and placed at 4°C.

A typical 1.2L cell culture would require 23 anion exchanges, resulting in 8.45mL of ~ 20g/L of concentrated protein. This would give a total yield of ~ 169g or 140mg/L culture.

The purification of DsRed2 was monitored by the Agilent Bioanalyzer 2100, which replicates and quantifies gel electrophoresis for proteins. The Agilent Protein 230 reagent kit (containing protein chips, electrode cleaner, a syringe, spin filters, gel-matrix, dye concentrate, sample buffer, and protein ladder) was used for the sizing range 14-230 kDa. Examples of Bioanalyzer outputs are in Appendix D.

DsRed2 Surfactant Experiment

The aqueous phase consisted of 0.5g/L DsRed2, 0.2M NaCl, 50mM carbonate buffer, pH~9. The organic phase consisted of 25 to 200mM cetyltrimethylammonium bromide (CTAB, Sigma, H9151) in 90% isooctane/10% hexanol (by volume) with 150uL H_2O/10mL organic phase.

DsRed2 Salt Experiment

The aqueous phase consisted of 0.5g/L DsRed2, 50mM carbonate buffer, pH~9, 0 to 0.75M NaCl. The organic phase consisted of 100mM CTAB in 90% isooctane/10% hexanol (by volume) with 150uL H_2O/10mL organic phase.

DsRed2 pH Experiment

The aqueous phase consisted of 0.5g/L DsRed2, 0.1M NaCl, 50mM buffer, and pH~6-10.5. The organic phase consisted of 100mM CTAB in 90% isooctane/10% hexanol (by volume) with 150uL H_2O/10mL organic phase.

DsRed2 Analysis

The analysis was the same as for cytochrome c, except that the absorbance was read at 563 nm. The blank for the AOT micelles was isooctane and the blank for the CTAB micelles was 90% isooctane/10% hexanol.

Statistical Analysis

Each RM experiment was performed three times (runs), with the same starting solutions. The results were analyzed by UV-visible spectroscopy only once. For statistical analysis, the results of the three runs for an experiment were averaged and the standard deviation was found by using the stdev() function in Excel Office. The averages were then graphed and the standard deviation was used for both the positive and negative error bars on the graph.

Far-UV CD

The circular dichroic measurements were taken with a Jasco J-810 CD spectropolarimeter in a 0.1cm round glass cuvette. The settings were as follows: high sensitivity, 260 to 190nm, 1nm pitch, continuous scanning mode, 50 nm/min scanning speed, 16msec response, 5nm bandwidth, and 80-150 accumulation. The measurements of a blank aqueous solution (0.2M NaCl, 1mM phosphate buffer) were subtracted from the measurements of an aqueous solution of DsRed2 (15μM protein, 0.2M NaCl, 1mM phosphate buffer). The measurements of an empty micelle organic solution (resulting from 0.2M NaCl, 1mM phosphate buffer contacted with 100mM CTAB in 90% isooctane/10% hexanol) were subtracted from the measurements of DsRed2 micelles (resulting from 15μM protein, 0.2M NaCl, 1mM phosphate buffer contacted with 100mM CTAB in 90% isooctane/10% hexanol). Both sets of data were subjected to the means

movement smoothing algorithm with 5-7 convolution width. The y-axis was converted from mdeg to molecular ellipticity.

CHAPTER IV

Results

Only the most recent, successful experiments are described here. Several examples of earlier, less successful experiments are presented in Appendix C, as well as a second method of analysis using the Agilent Bioanalyzer 2100. No conclusions will be drawn from these data. All raw data for the described experiments below are presented in Appendix A.

Cytochrome c with AOT

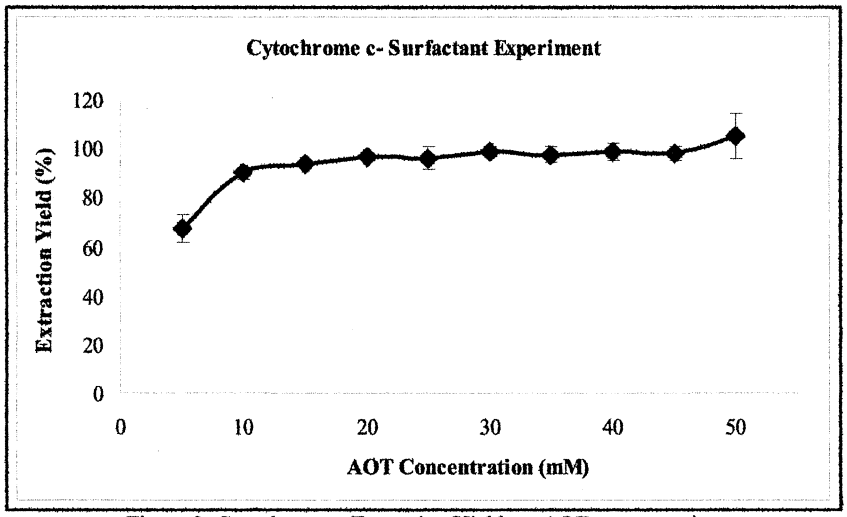

Figure 2. Cytochrome c Extraction Yield vs. AOT concentration
(aqueous: 0.5g/L cyt c, 0.3M NaCl, 0.02M Tris buffer, pH~9.7, organic: 5 to 50mM AOT in isooctane)

Cytochrome c is easily extracted into reverse micelles at low concentrations of surfactant. As shown in Figure 2, greater than 90% of cytochrome c was transferred above 5mM AOT.

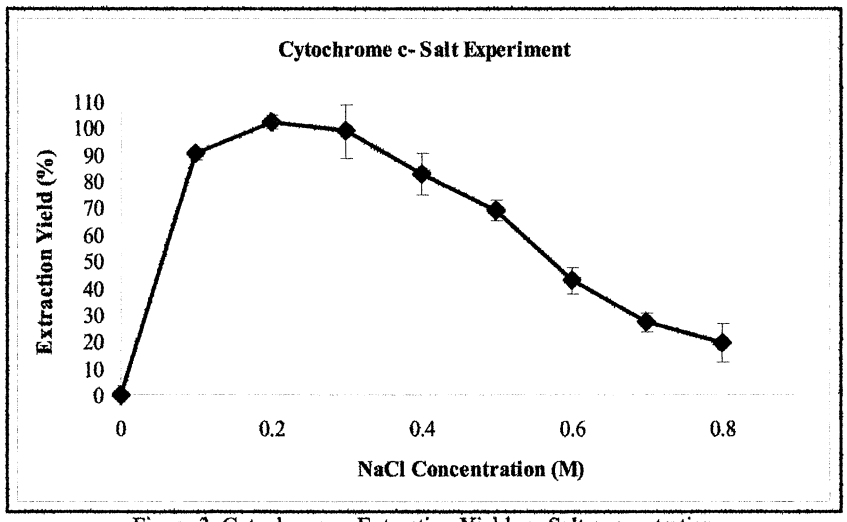

Figure 3. Cytochrome c Extraction Yield vs. Salt concentration
(aqueous: 0.5g/L cyt c, 0.02M Tris buffer, pH~9.7, 0 to 0.8M NaCl, organic: 50mM AOT in isooctane)

As expected, cytochrome c was not transferred without salt. Low concentrations (0.1-0.3M) are needed for greater than 90% yield, with 0.2M having the highest yield. Higher than 0.3M, the extraction yield decreases dramatically.

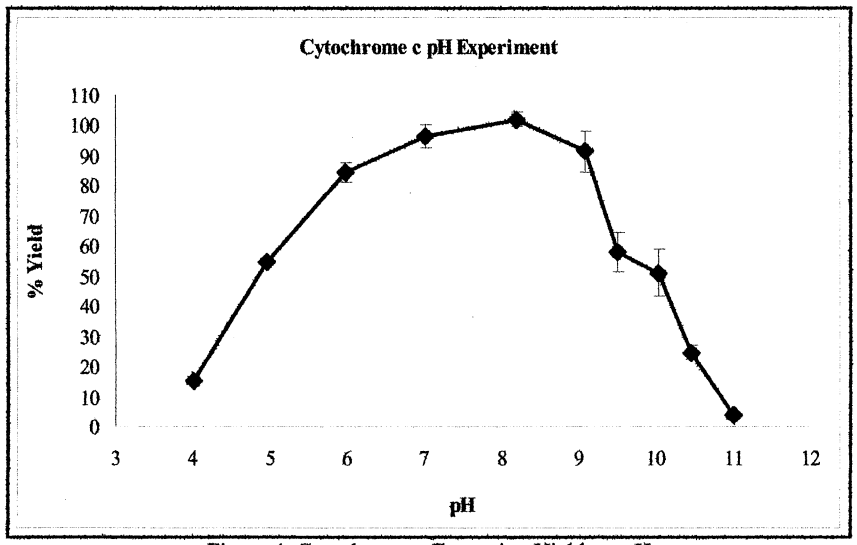

Figure 4. Cytochrome c Extraction Yield vs. pH
(aqueous: 0.5g/L cyt c, 0.3M NaCl, 50mM buffer, pH 4-11, organic: 50mM AOT in isooctane)

Cytochrome c was over 90% extracted in the pH range 7-8. The protein was extracted over 80% in the pH range 6-9, but decreased outside of this range.

DsRed2 with AOT

DsRed2 did not transfer with AOT from pH 4.5 -11 with surfactant concentrations 50, 200, and 500 mM.

DsRed2 with CTAB

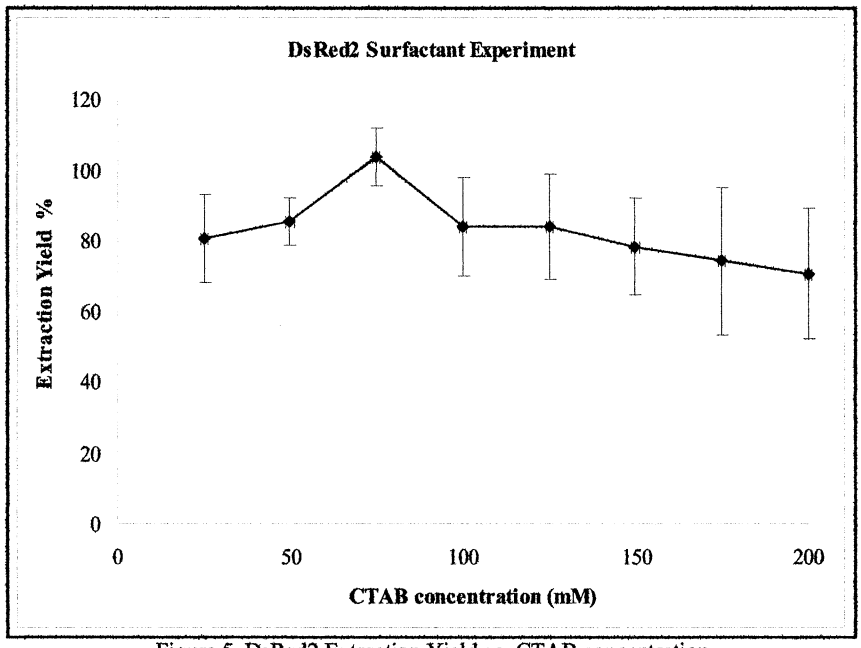

Figure 5. DsRed2 Extraction Yield vs. CTAB concentration
(aqueous: 0.5g/L DsRed2, 0.2M NaCl, 50mM carbonate buffer, pH~9, organic: 25 to 200mM CTAB in 90% isooctane/10% hexanol)

Over 80% of DsRed2 was extracted with surfactant concentrations up to 125mM CTAB, with the highest extraction yield occurring at 75mM.

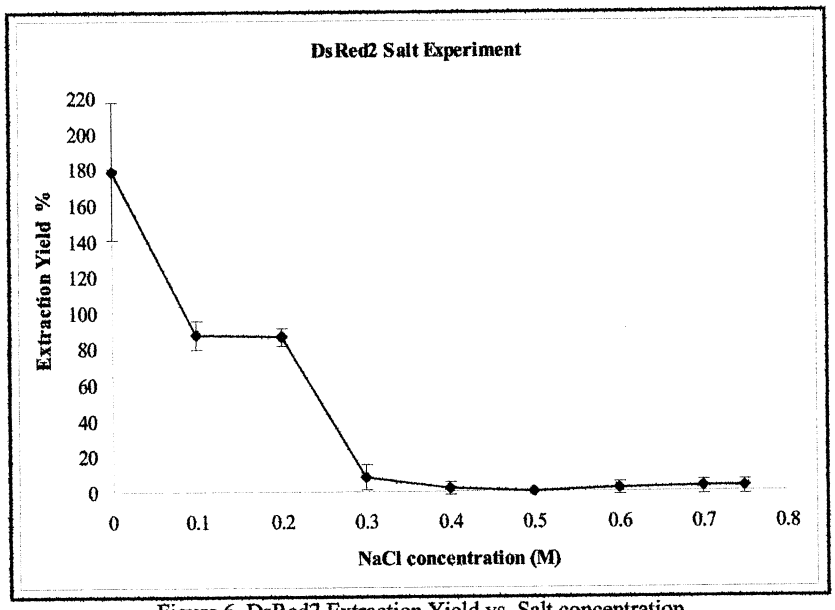

Figure 6. DsRed2 Extraction Yield vs. Salt concentration
(aqueous: 0.5g/L DsRed2, 50mM carbonate buffer, pH~9, 0 to 0.75M NaCl, organic: 100mM CTAB in 90% isooctane/10% hexanol)

Over 80% of DsRed2 was extracted with salt concentrations of 0.1M and 0.2M. The extraction yield decreased greater than 0.2M NaCl. The micelles were not stable at 0M NaCl; the organic phases were cloudy, causing absorbances higher than 100%.

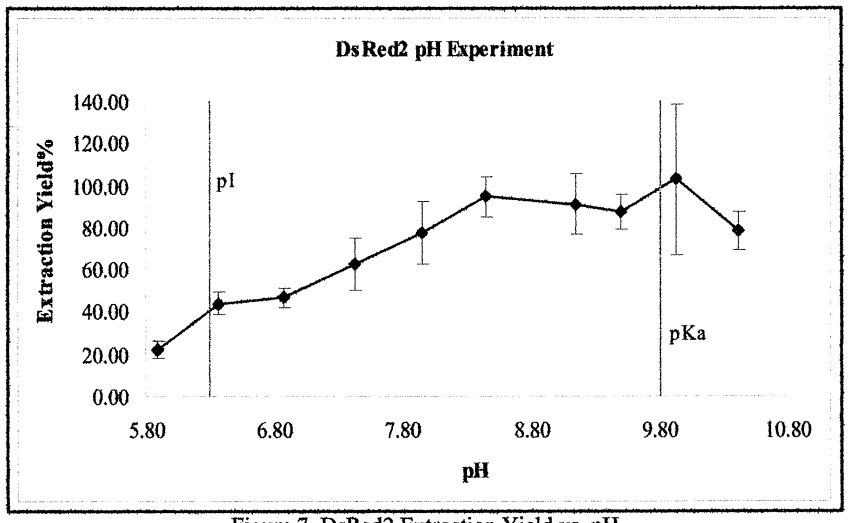

Figure 7. DsRed2 Extraction Yield vs. pH
Vertical lines: pI of DsRed2 = 6.3, pKa of CTAB surfactant = 9.81[69]
(aqueous: 0.5g/L DsRed2, 0.1M NaCl, 50mM buffer, pHs 5.9-10.4, organic: 100mM CTAB in 90% isooctane/10% hexanol)

In general, the extraction yield increased with increasing pH. Above 80% of the protein was extracted from pH 8.5 – 9.9. The highest extraction occurred at pH 9.9, although this point has a large amount of error.

Far-UV CD

Figure 8 below shows far-UV CD data of aqueous cytochrome c. The data show a typical CD spectrum for an α-helical protein. It has a positive band at 197nm and negative bands at 210 and 222nm.

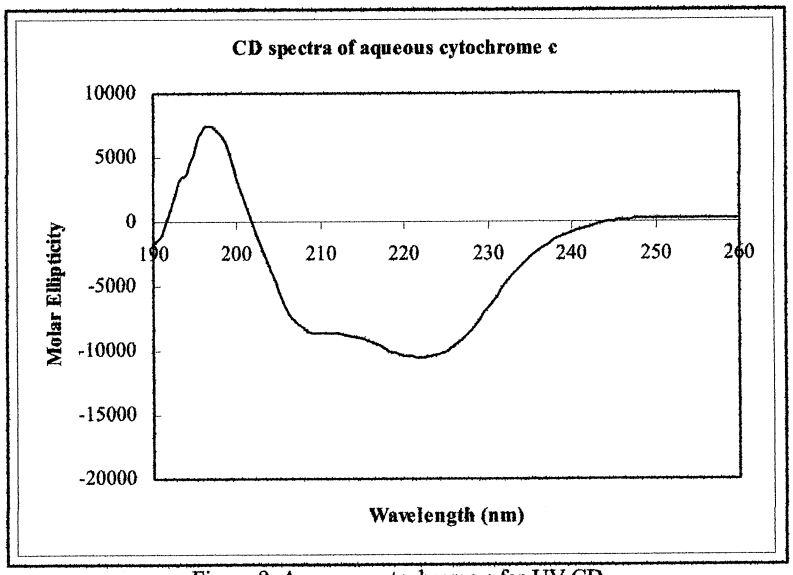

Figure 8. Aqueous cytochrome c far-UV CD
(aqueous: 28.2μM (0.37g/L) cyt c, 0.2M NaCl, 5mM phosphate buffer)

Figure 9 below shows far-UV CD data of cytochrome c in reverse micelles. The spectrum still has characteristics of α-helices, but is drastically different from Figure 8, indicating changes in the secondary structure upon solubilization in RMs.

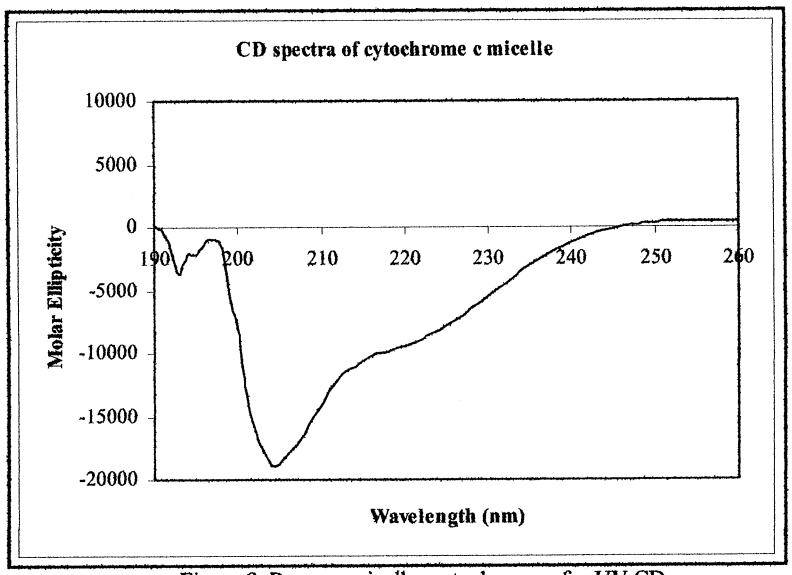

Figure 9. Reverse micellar cytochrome c far-UV CD
(aqueous: 28.2μM (0.37g/L) cyt c, 0.2M NaCl, 5mM phosphate buffer, organic: 50mM AOT in isooctane, resulting RM has 16.7μM (0.21g/L) cyt c)

The CD spectra in Figures 8 and 9 were analyzed by CDPro software[70] using three methods of analysis: SELCON3, CONTINLL, and CDSSTR. The individual results from this software and the average are in Table 2 below where H is helical, S is strand, Trn is turn, UnRd is unordered, r is regular, and d is distorted. The raw data is in Appendix B.

Table 2. Analysis of Cytochrome c CD Data

Structure		*H(r)*	*H(d)*	*S(r)*	*S(d)*	*Trn*	*UnRd*	*Sum*
Aqueous cyt c	SELCON3	0.113	0.138	0.146	0.077	0.218	0.325	1.017
	CDSSTR	0.103	0.141	0.158	0.085	0.254	0.264	1.005
	CONTINLL	0.113	0.128	0.138	0.075	0.218	0.328	1.000
	Average	0.110 ± 0.006	0.136 ± 0.007	0.147 ± 0.010	0.079 ± 0.005	0.230 ± 0.021	0.306 ± 0.036	1.008
RM cyt c	SELCON3	0.163	0.203	0.028	0.045	0.222	0.312	0.973
	CDSSTR	0.231	0.226	0.093	0.061	0.150	0.240	1.001
	CONTINLL	0.130	0.185	0.097	0.045	0.233	0.310	1.000
	Average	0.175 ± 0.052	0.205 ± 0.021	0.073 ± 0.039	0.050 ± 0.009	0.202 ± 0.045	0.287 ± 0.041	0.992

Figure 10 below shows far-UV CD data of aqueous DsRed2. The data show a typical CD spectrum for a β-strand protein: there is a positive band at 201nm and a negative band at 222nm.

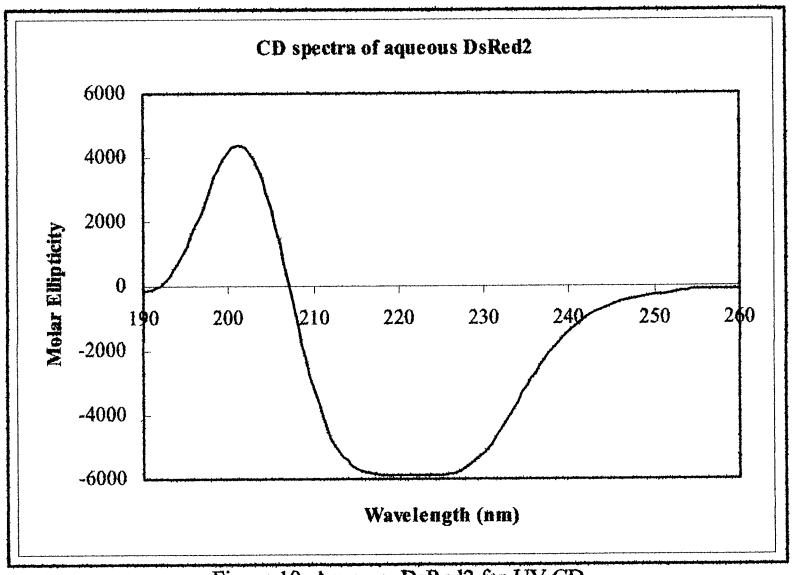

Figure 10. Aqueous DsRed2 far-UV CD
(aqueous: 15.3μM (1.6g/L) DsRed2, 0.2M NaCl, 1mM phosphate buffer)

Figure 11 below shows far-UV CD data of reverse micellar DsRed2. The graph still has characteristics of β-strands, but is drastically different from Figure 10, indicating changes in the secondary structure upon solubilization in RMs.

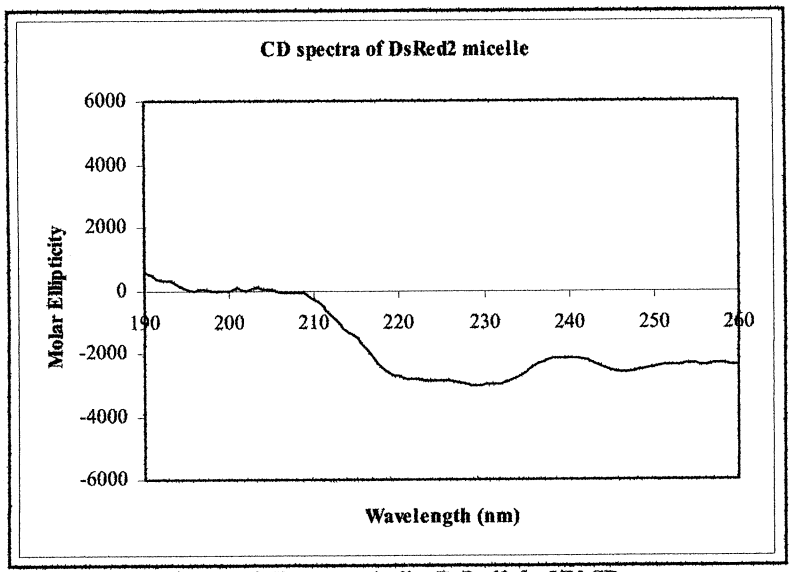

Figure 11. Reverse micellar DsRed2 far-UV CD
(aqueous: 15.3μM (1.6g/L) DsRed2, 0.2M NaCl, 1mM phosphate buffer, organic: 100mM CTAB in 90% isooctane/10% hexanol, resulting RM has 8.4μM (0.87g/L) DsRed2

The CD spectra in Figures 10 and 11 were also analyzed by CDPro software using three methods of analysis: SELCON3, CONTINLL, and CDSSTR. The individual results from this software and the average are in Table 3 with the same abbreviations as Table 2. The raw data is in Appendix B.

Table 3. Analysis of DsRed2 CD Data

Structure		*H(r)*	*H(d)*	*S(r)*	*S(d)*	*Trn*	*UnRd*	*Sum*
Aqueous DsRed2	SELCON3	0.049	0.057	0.230	0.122	0.231	0.342	1.031
	CDSSTR	0.006	0.036	0.282	0.123	0.249	0.303	0.999
	CONTINLL	0.026	0.017	0.221	0.098	0.292	0.346	1.000
	Average	0.027 ± 0.022	0.037 ± 0.020	0.244 ± 0.033	0.114 ± 0.014	0.257 ± 0.031	0.330 ± 0.024	1.010
RM DsRed2	SELCON3	0.041	0.028	0.262	0.138	0.191	0.368	1.028
	CDSSTR	-0.002	0.026	0.281	0.130	0.246	0.304	0.985
	CONTINLL	0.007	0.053	0.286	0.140	0.221	0.294	1.001
	Average	0.015 ± 0.023	0.036 ± 0.015	0.276 ± 0.013	0.136 ± 0.005	0.219 ± 0.028	0.322 ± 0.040	1.005

CHAPTER V

Discussion

Cytochrome c

The results here will be compared to 4 studies on cytochrome c extraction with AOT reverse micelles: Connell[60], Göklen et al.[27], Ichikawa et al.[32], and Jarudilokkul et al.[41]

The surfactant experiment supports the trend in previous studies that the extraction yield of cytochrome c increases as the surfactant concentration increases. An increase in surfactant concentration increases the driving force. Ichikawa et al. reported that the minimum AOT concentration for 100% extraction of cytochrome c was 10mM for 0.5g/L protein and 15mM for 1g/L with pH 7.2-8.2 and 0.1M KCl. Connell reported that the minimum AOT concentration for over 70% extraction was 20mM at 1g/L and 10mM for 0.22g/L at pH 9.7 and 0.1M Na^+. For both studies, the lower protein concentration required less surfactant for extraction, as expected. However, the large standard deviations in Connell's study hint that 0.1M ionic concentration may not be enough for micelle stabilization.

Jarudilokkul et al. reported that the minimum AOT concentration for over 90% extraction was 16mM at pH 10, which is a higher concentration than reported here. This can be explained by the aqueous conditions of both studies. Jarudilokkul et al. had double the protein concentration (1g/L cyt c) and lower ionic concentration (80% 0.1M NaCl and 20% 0.1M buffer for total Na^+ of 0.1M). Since this study had 0.5g/L cyt c, a lower

concentration of AOT is needed, leading to a minimum AOT concentration (for over 90% extraction) at 5mM at pH 9.7.

The salt experiment supports the trend in previous studies that at low ionic strengths, cytochrome c is extracted, but at high ionic strengths, the extraction yield of the protein decreases substantially due to a disruption of electrostatic interactions. Göklen et al. transferred 1g/L cytochrome c with 0.1M KCl and back-transferred with a fresh aqueous phase of 1.0M KCl. Ichikawa et al. saw a substantial decrease in extraction yield in transferring 1g/L cytochrome c with greater than 0.2M NaCl. Connell reported a greater than 90% extraction yield for 0.25g/L cytochrome c (0.02M Tris, pH 9.7) with 0.24M NaCl and greater than 80% extraction for 0.16 – 0.40M NaCl. This study agrees well with Connell's study because for this study, the highest extraction yield was at 0.2M NaCl and there was greater than 80% extraction for 0.1- 0.4M NaCl.

The pH experiment supports the literature that very little protein is extracted greater than pH 10, as the pI of cytochrome c is 10.6. Greater than 10.6, cytochrome c is negatively charged and will not transfer with an anionic surfactant. The polar head group of the surfactant (sulfonic acid) has a pKa less than 0.7[71], and was negatively charged for the ranges of pH used in the experiment. Jarudilokkul et al. reported that the optimum pH for extracting 1g/L of cytochrome c is 10 and that greater than 90% of the protein transferred in the pH range 5 to 11. Ichikawa et al. reported that extraction of 1g/L cytochrome c decreased at pHs higher than 10 and that 90% of the protein transferred in the pH range 6 to 10. Connell reported that 80% of 0.2g/L cytochrome c transferred at pH 7.8. This thesis reports that greater than 80% of 0.5g/L of cytochrome c is transferred in the pH range 6 -9 and that 90% transferred in the range 7 – 9, which is a slightly lower

profile than previous studies. This could be because this study uses a higher salt concentration than the other studies, causing a narrower pH profile.

DsRed2

DsRed2 did not extract with the anionic surfactant AOT. For the protein to be extracted by electrostatic interactions, DsRed2 must be in a pH environment much less than its pI, 6.3. However, the protein loses its fluorescence and is not stable below pH 4.5.[72,73] This narrow pH window (4.5-6.3) does not allow for strong enough electrostatic interactions to extract DsRed2 into an AOT micelle. For the protein to be extracted above its pI, there must be a strong hydrophobic driving force, which did not occur. Therefore, DsRed2 could not be extracted by electrostatic or hydrophobic interactions into the AOT/isooctane system in this thesis. A previous study[59] was able to extract DsRed from a dilute aqueous solution (0.05mg/ml protein, 50mM Tris-HCl buffer, pH 8) into an AOT micelle by the injection method. The success of this study in extracting DsRed may be due to the difference between DsRed and DsRed2, or between the injection and phase-transfer methods.

For DsRed2 extraction with CTAB, the results here will be compared to one similar study (Connell[60]). For DsRed2 extraction with varying surfactant concentration, Connell reported no minimum surfactant concentration, as all DsRed2 was over 90% extracted from 5-100mM CTAB in 90% isooctane/10% hexanol. Connell's aqueous phase for the surfactant experiments consisted of 2.4g/L protein, 50mM carbonate buffer, pH ~9, and no salt. The results here compare well with Connell. Over 80% of DsRed2 was extracted with surfactant concentrations up to 125mM. Higher than 125mM, the

extraction yield decreased slightly to within 70-80% yield, perhaps due to size exclusion effects.

For DsRed2 extraction varying salt concentration, Connell reported over 80% extraction below 0.2M NaCl. The extraction yield dropped to 40% at 0.25M, and decreased rapidly at higher salt concentrations. The results here compare well with Connell, despite the instability of the micelles with no salt. Over 80% extraction was achieved with salt concentrations of 0.2M and below. The extraction yield dropped dramatically with higher salt concentrations. The results of Connell's study and this thesis indicate that 0.2M NaCl is the maximum salt concentration for DsRed2 extraction into a reverse micelle.

For DsRed2 extraction varying pH, Connell reported greater than 80% extraction in the pH range 7-10.5, with pH 9 having the highest yield. This study reports a narrower extraction profile in that over 80% of DsRed2 was extracted in the pH range 8.5-9.9. This result can be explained by the salt concentration. Connell did not use salt in the aqueous phase of the pH experiment, while 0.1M NaCl was used here. The presence of salt screened the electrostatic interactions between the negatively-charged DsRed2 (above pH 6.3) and the cationic surfactant, leading to a narrower pH profile here. Also, the pKa of the head group of the surfactant (trimethylamine) is 9.81, which influenced the extraction yield of the protein at pHs close to and greater than the pKa. The large standard error at pH 9.9 can be explained by the proximity to the pKa of the surfactant. Greater than the pKa, the surfactant will be negatively charged and the electrostatic interactions between the charged head group and the protein will be reduced.

Further Analysis

As stated earlier, the type and concentration of buffer can affect the solubilization of proteins into reverse micelles. Data were pulled from the 3 extraction profiles (surfactant, salt, pH) and CD to analyze the effects of the buffer on extraction yield for both cytochrome c and DsRed2.

Figure 12 below shows the effect of buffer type and concentration on extraction yield for cytochrome c. Table 4 summarizes the pulled data in Figure 12. The two points with the lowest extraction yields have, interestingly, the lowest and highest buffer concentrations. The point at 5mM buffer may have a low yield because the salt concentration was too low, despite having a favorable pH, protein concentration, and buffer concentration. The point at 50mM buffer with the lower extraction yield may have a low yield because the buffer concentration is too high or the pH is slightly unfavorable. Therefore, no direct conclusions can be drawn from the graph since each point has dissimilar conditions (salt, pH, protein concentration, etc.). However, it would be advantageous to conduct an experiment altering only the concentration and type of buffer to determine the direct influence on the extraction yield.

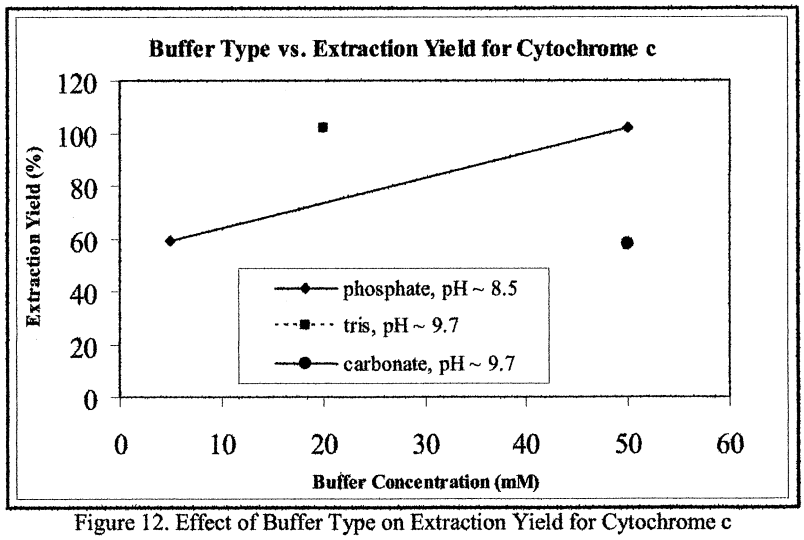

Figure 12. Effect of Buffer Type on Extraction Yield for Cytochrome c

Table 4. Pulled Data for Effect of Buffer Type on Extraction Yield for Cytochrome c

Experiment	*Buffer Type*	*Buffer Concentration (mM)*	*Salt Concentration (M)*	*pH*	*Protein Concentration (g/L)*	*Extraction Yield %*	*Standard Deviation*
CD	Phosphate	5	0.2	8.5	0.37	59.15	n/a
Surfactant & Salt, Averaged	Tris	20	0.3	9.7	0.50	102.27	9.20
pH	Phosphate	50	0.3	8.2	0.5	102.04	2.53
pH	Carbonate	50	0.3	9.5	0.5	57.94	6.6

Figure 13 shows the effect of buffer type and concentration on extraction yield for DsRed2. Table 5 summarizes the pulled data in Figure 13. As with Figure 12, no conclusions can be drawn from Figure 13 without further investigation.

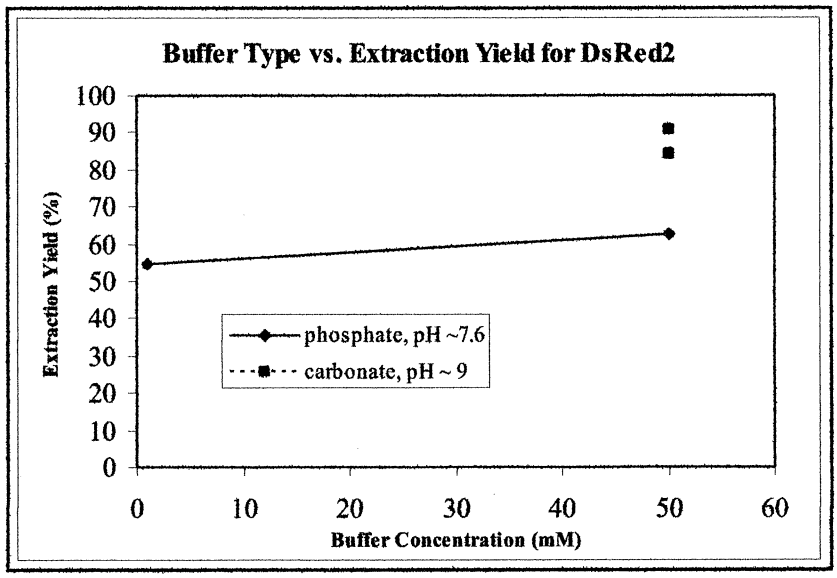

Figure 13. Effect of Buffer Type on Extraction Yield for DsRed2

Table 5. Pulled Data for Effect of Buffer Type on Extraction Yield for DsRed2

Experiment	*Buffer Type*	*Buffer Concentration (mM)*	*Salt Concentration (M)*	*pH*	*Protein Concentration (g/L)*	*Extraction Yield %*	*Standard Deviation*
CD	Phosphate	1	0.2	7.6	1.6	54.89	n/a
Surfactant & Salt, Averaged	Carbonate	50	0.2	9	0.5	84.34	9.72
pH	Phosphate	50	0.1	7.43	0.5	62.62	12.38
pH	Carbonate	50	0.1	9.15	0.5	90.83	14.23

Far-UV CD

The CD spectra of the micellar solutions of both cytochrome c and DsRed2 are very different from the aqueous CD spectra, but still retain characteristics of their respective secondary structure (α-helical or β-strand). This difference may be due to a change in the secondary structure of the proteins after solubilization or interference from the micelle structure, which has been noted in previous studies.[18] It would be informative to perform CD studies on the aqueous phase after back-transfer, to determine if any structure change is permanent.

Analysis of the data by CDPro software indicates that cytochrome c increases in helical content upon solubilization. Since the protein is known to solubilize at the interior interface of RMs, the protein's interactions with the polar head groups of the surfactant and the enclosed space of the water pool may force the protein into a tighter structure. Also, the increase in helical content determined by the software can be explained by the addition of two structures: an α-helical and an extended/denatured protein, which would result in a graph similar to Figure 9. It has previously been shown that cytochrome c denatures in AOT reverse micelles,[17,74,75] so this is most likely the case here. If the W_0 had been determined for the RMs in Figure 9, the far-UV CD results could be directly compared to those of Naoe et al.[17]

For DsRed2, analysis of the data by CDPro software indicates that DsRed2 decreases the content of regular helical, turns, and unordered, but increases the content of strands (both regular and distorted). The distorted helical content remains the same. However, the accuracy of the analysis is to be taken into consideration, as the standard deviations are quite high, especially for helical content. Also, CDSSTR reported a negative content for regular helical, which is impossible. However, it can be stated that the CD data indicate some denaturing of DsRed2 in reverse micelles.

The denaturing of these proteins in reverse micelles may be reduced or prevented by altering the charged surfactant in order to decrease the electrostatic interactions between the polar head group of the surfactant and the charges on the protein. Wand et al.[76,77] reported using a short AOT analogue and binary and tertiary mixtures of anionic, cationic, and nonionic surfactants to maintain the structural fidelity of cytochrome c and

other proteins in reverse micelles. Doussin et al.[78] reported synthesizing a zwitterionic surfactant that increases the amount of time cytochrome c remains stable in RMs.

CHAPTER VI

Conclusion

Cytochrome c and DsRed2 were successfully extracted into reverse micelles by the contacting of an aqueous protein-containing phase with an organic phase. Two important properties that differentiate the extraction profiles of these proteins are pI and size. Cytochrome c is a relatively small, monomeric protein with a pI of 10.6. It was easily extracted into reverse micelles with the anionic surfactant AOT. DsRed2, however, is a large tetramer with a pI of 6.3. The protein could not be extracted into AOT, but was extracted with CTAB, and with a wider error range than cytochrome c. CD data indicate the secondary structure of the proteins may change with solubilization into reverse micelles, despite absorption interference from the micelles.

The results of this thesis suggest that extraction of certain proteins into reverse micelles is a viable primary separation step for the recombinant biotechnology industry. However, each process will have to be optimized to the protein of interest, as protein extraction is specific to certain properties and is extremely sensitive. Göklen and Hatton[27] noticed a shift in their data with a change in supply of either protein or surfactant. More scale-up research is needed before the technique can be successfully adapted in industry. By then, affinity surfactants will hopefully improve the extraction even further.[79-81] Also, natural surfactants such as lecithin will need to be studied for use in the food and pharmaceutical industries.[82]

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APPENDICES

APPENDIX A

Experimental Data

Table 6. Cytochrome c Surfactant Experiment Data, Organic Phase

AOT	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	St. Dev.
(mM)	A410	Yield %	A410	Yield %	A410	Yield %	A410	(M)	(g/L)	Yield %	
initial	6.95	n/a	7.086	n/a	7.132	n/a	7.056	6.66E-05	0.824	n/a	n/a
5	5.046	71.51	4.315	61.15	4.934	69.93	4.765	4.50E-05	0.557	67.53	5.58
10	6.207	87.97	6.55	92.83	6.314	89.48	6.357	6.00E-05	0.743	90.1	2.48
15	6.541	92.71	6.603	93.58	6.743	95.57	6.629	6.25E-05	0.774	93.95	1.47
20	6.734	95.44	6.881	97.52	6.794	96.29	6.803	6.42E-05	0.795	96.42	1.05
25	6.629	93.95	6.585	93.33	7.149	101.31	6.788	6.40E-05	0.793	96.2	4.44
30	6.795	96.31	6.981	98.94	7.109	100.76	6.962	6.57E-05	0.813	98.67	2.24
35	6.635	94.03	6.942	98.39	7.051	99.94	6.876	6.49E-05	0.803	97.45	3.06
40	6.677	94.63	7.182	101.79	7.057	100.01	6.972	6.58E-05	0.815	98.81	3.73
45	6.71	95.09	6.984	98.97	7.082	100.38	6.925	6.53E-05	0.809	98.15	2.74
50	8.123	115.12	6.837	96.9	7.271	103.05	7.41	6.99E-05	0.866	105.02	9.27

Table 7. Cytochrome c Salt Experiment Data, Organic Phase

NaCl	*Run #1*		*Run#2*		*Run#3*		*Average*	*Conc.*	*Conc.*	*Avg.*	*St. Dev.*
(M)	*A410*	*Yield %*	*A410*	*Yield %*	*A410*	*Yield %*	*A410*	*(M)*	*(g/L)*	*Yield %*	
initial	6.849	n/a	7.103	n/a	6.825	n/a	6.925	6.53E-05	0.809	n/a	n/a
0	0	0	0	0	0.012	0.18	0.004	3.86E-08	0	0.06	0.1
0.1	6.146	88.74	6.447	93.1	6.294	90.88	6.296	5.94E-05	0.736	90.91	2.18
0.2	7.297	105.37	6.927	100.03	7.111	102.68	7.112	6.71E-05	0.831	102.69	2.67
0.3	7.702	111.22	6.518	94.11	6.456	93.23	6.892	6.50E-05	0.805	99.52	10.14
0.4	5.769	83.3	5.207	75.19	6.325	91.34	5.767	5.44E-05	0.674	83.28	8.07
0.5	4.611	66.58	4.709	67.99	5.135	74.15	4.818	4.55E-05	0.563	69.57	4.03
0.6	2.781	40.15	2.75	39.71	3.391	48.97	2.974	2.81E-05	0.347	42.94	5.22
0.7	1.625	23.46	1.962	28.33	2.088	30.15	1.891	1.78E-05	0.221	27.31	3.46
0.8	0.972	14.03	1.199	17.32	1.939	28	1.37	1.29E-05	0.16	19.78	7.3

Table 8. Cytochrome c pH Experiment Data, Organic Phase

pH	Initial	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	
	A410	A410	Yield %	A410	Yield %	A410	Yield %	A410	(M)	(g/L)	Yield %	St. Dev.
4.005	6.006	0.898	14.95	0.934	15.55	0.942	15.69	0.925	8.73E-06	0.108	15.4	0.39
4.961	6.497	3.496	53.81	3.54	54.48	3.635	55.95	3.557	3.36E-05	0.416	54.75	1.09
5.988	6.86	5.572	81.22	5.83	84.99	5.987	87.28	5.796	5.47E-05	0.677	84.5	3.06
7.004	6.647	6.26	94.17	6.251	94.04	6.7	100.79	6.404	6.04E-05	0.748	96.33	3.86
8.203	6.473	6.609	102.09	6.768	104.54	6.44	99.49	6.606	6.23E-05	0.772	102.04	2.53
9.093	6.875	6.019	87.56	6.028	87.68	6.817	99.17	6.288	5.93E-05	0.735	91.47	6.67
9.506	6.64	3.535	53.23	3.659	55.1	4.349	65.49	3.847	3.63E-05	0.449	57.94	6.6
10.026	6.824	3.05	44.69	3.344	49	4.086	59.87	3.493	3.30E-05	0.408	51.19	7.82
10.459	6.642	1.432	21.56	1.724	25.96	1.755	26.42	1.637	1.54E-05	0.191	24.64	2.68
11.009	7.045	0.24	3.4	0.262	3.72	0.313	4.44	0.272	2.56E-06	0.032	3.86	0.53

Table 9. DsRed2 Surfactant Experiment Data, Organic Phase

CTAB	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	Std. Dev.
(mM)	A563	Yield %	A563	Yield %	A563	Yield %	A563	(M)	(g/L)	Yield %	
Initial	0.093	n/a	0.095	n/a	0.092	n/a	0.094	2.14E-06	0.22	n/a	n/a
25	0.078	83.85	0.062	66.53	0.085	91.12	0.075	1.72E-06	0.177	80.5	12.63
50	0.087	92.5	0.073	97.44	0.08	105.79	0.08	1.82E-06	0.188	85.36	6.72
75	0.116	123.55	0.088	110.2	0.087	108.88	0.097	2.21E-06	0.228	103.52	8.12
100	0.091	97.54	0.076	78.93	0.068	70.05	0.079	1.79E-06	0.185	83.92	14.03
125	0.075	80.31	0.087	110.36	0.073	93.38	0.078	1.79E-06	0.184	83.76	15.07
150	0.071	76.43	0.081	103.7	0.067	85.47	0.073	1.67E-06	0.172	78.29	13.89
175	0.061	65.29	0.077	105.74	0.069	94.79	0.069	1.58E-06	0.163	74.1	20.93
200	0.063	66.97	0.072	103.32	0.064	92.11	0.066	1.51E-06	0.155	70.59	18.61

Table 10. DsRed2 Salt Experiment Data, Organic Phase

NaCl (M)	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	Std. Dev.
	A563	Yield %	A563	Yield %	A563	Yield %	A563	(M)	(g/L)	Yield %	
Initial	0.289	n/a	0.307	n/a	0.33	n/a	0.309	7.05E-06	0.726	n/a	n/a
0	0.572	197.83	0.634	206.81	0.449	136	0.552	1.26E-05	1.297	178.75	38.55
0.1	0.245	84.86	0.297	96.71	0.271	81.93	0.271	6.18E-06	0.637	87.74	7.82
0.2	0.263	91.09	0.268	87.29	0.268	81.13	0.266	6.08E-06	0.626	86.28	5.02
0.3	0	0	0.044	14.27	0.03	9.15	0.025	5.63E-07	0.058	7.99	7.23
0.4	0	0	0.019	6.26	0	0	0.006	1.46E-07	0.015	2.07	3.61
0.5	0	0	0	0	0	0	0	0.00E+00	0	0	0
0.6	0	0	0.017	5.66	0	0	0.006	1.32E-07	0.014	1.87	3.27
0.7	0.021	7.22	0	0	0	0	0.007	1.59E-07	0.016	2.25	4.17
0.75	0	0	0.021	6.77	0	0	0.007	1.58E-07	0.016	2.24	3.91

Table 11. DsRed2 pH Extraction Data, Organic Phase

pH	Initial	Run #1		Run#2		Run#3		Avg.	Conc.	Conc.	Avg.	Std. Dev.
	A563	A563	Yield %	A563	Yield %	A563	Yield %		(M)	(g/L)	Yield %	
5.9	0.294	0.061	20.65	0.079	26.93	0.055	18.71	0.065	1.48E-06	0.153	22.09	4.29
6.37	0.309	0.14	45.29	0.15	48.46	0.117	37.81	0.136	3.10E-06	0.319	43.85	5.47
6.88	0.329	0.146	44.42	0.145	43.91	0.17	51.57	0.154	3.51E-06	0.361	46.63	4.28
7.43	0.348	0.195	56.08	0.191	54.88	0.268	76.89	0.218	4.98E-06	0.513	62.62	12.38
7.96	0.336	0.242	72.14	0.223	66.42	0.316	94.23	0.261	5.95E-06	0.613	77.6	14.69
8.45	0.335	0.284	84.89	0.346	103.35	0.318	94.96	0.316	7.21E-06	0.743	94.4	9.25
9.15	0.34	0.271	79.67	0.292	85.95	0.364	106.86	0.309	7.06E-06	0.727	90.83	14.23
9.5	0.331	0.273	82.4	0.275	82.86	0.32	96.63	0.289	6.60E-06	0.68	87.3	8.09
9.94	0.338	0.28	82.89	0.273	80.86	0.488	144.3	0.347	7.93E-06	0.816	102.68	36.05
10.42	0.352	0.303	85.93	0.281	79.8	0.242	68.6	0.275	6.28E-06	0.647	78.11	8.79

APPENDIX B

CDPro Data

CDPro Data for aqueous cytochrome c

SAMPLE: : cytochrome c aqueous phase
PROGRAM: : SELCON3
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	:	.113	.138	.146	.077	.218	.325

RMSD(Exp-Calc): .771
NRMSD(Exp-Cal): .364

SAMPLE: :cytochrome c aqueous phase
PROGRAM: : CDSSTR
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	:	.103	.141	.158	.085	.254	.264

RMSD(Exp-Calc): .198
NRMSD(Exp-Cal): .093

SAMPLE: : cytochrome c aqueous phase
PROGRAM: : CONTINLL
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Turn	Unrd
Fractions	:	.113	.128	.138	.075	.218	.328

RMSD(Exp-Calc): .123
NRMSD(Exp-Cal): .058

CDPro Data for reverse micellar cytochrome c

SAMPLE: : cyt c micelle
PROGRAM: : SELCON3
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	.163	.203	.028	.045	.222	.312

RMSD(Exp-Calc): 1.664
NRMSD(Exp-Cal): .571

SAMPLE: :cyt c micelle
PROGRAM: : CDSSTR
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	.231	.226	.093	.061	.150	.240

RMSD(Exp-Calc): .295
NRMSD(Exp-Cal): .101

SAMPLE: : cyt c micelle
PROGRAM: : CONTINLL
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Turn	Unrd
Fractions	.130	.185	.097	.045	.233	.310

RMSD(Exp-Calc): .291
NRMSD(Exp-Cal): .100

CDPro Data for aqueous DsRed2

SAMPLE: : dsred2 aqueous
PROGRAM: : CONTINLL
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Turn	Unrd
Fractions	:	.026	.017	.221	.098	.292	.346

RMSD(Exp-Calc): .083
NRMSD(Exp-Cal): .066

SAMPLE: :dsred2 aqueous
PROGRAM: : CDSSTR
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	:	.006	.036	.282	.123	.249	.303

RMSD(Exp-Calc): .137
NRMSD(Exp-Cal): .110

SAMPLE: : dsred2 aqueous
PROGRAM: : SELCON3
Ref. Prot. Set: SMP56

Structure	:	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	:	.049	.057	.230	.122	.231	.342

RMSD(Exp-Calc): .533
NRMSD(Exp-Cal): .425

CDPro Data for reverse micellar DsRed2

SAMPLE: : dsred2 micelle
PROGRAM: : CONTINLL
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Turn	Unrd
Fractions	.007	.053	.286	.140	.221	.294

RMSD(Exp-Calc): .079
NRMSD(Exp-Cal): .137

SAMPLE: : dsred2 micelle
PROGRAM: : SELCON3
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	.041	.028	.262	.138	.191	.368

RMSD(Exp-Calc): .435
NRMSD(Exp-Cal): .753

SAMPLE: :dsred2 micelle
PROGRAM: : CDSSTR
Ref. Prot. Set: SMP56

Structure	H(r)	H(d)	S(r)	S(d)	Trn	Unrd
Fractions	-.002	.026	.281	.130	.246	.304

RMSD(Exp-Calc): .160
NRMSD(Exp-Cal): .277

APPENDIX C

Previous Experiments

One previous experiment for cytochrome c varying surfactant concentrations will be described here. This experiment was not discussed in the Results section of the thesis as no conclusions were drawn from the data. There are many more previous experiments that will not be described here because the data are also unusable and no conclusions were drawn from them also.

For the experiment below, the absorbances of both the organic and aqueous phases were measured on a UV-visible spectropolarimeter. It was determined that the aqueous phases had surfactant contamination, and therefore all experiments in the Results section analyzed only the organic phases.

A second method of analysis, electrophoresis by Agilent Bioanalyzer 2100, was used for the experiment below for comparison with the absorbance data. This second method of analysis was not comparable to the spectropolarimeter; later experiments only used a UV-visible spectropolarimeter to analyze data.

Cytochrome c Surfactant Experiment- Absorbance Data

Figures 14 and 15 below show the extraction profiles for cytochrome c varying surfactant concentration using absorbance data from the organic and aqueous phases, respectively. Tables 12 and 13 summarize the data. The aqueous phases had contamination from the surfactant, causing higher absorbances. Also, the aqueous and

organic phases do not equal ~100% because of the contamination. Therefore, only the organic phases of the experiments in the Results section were analyzed.

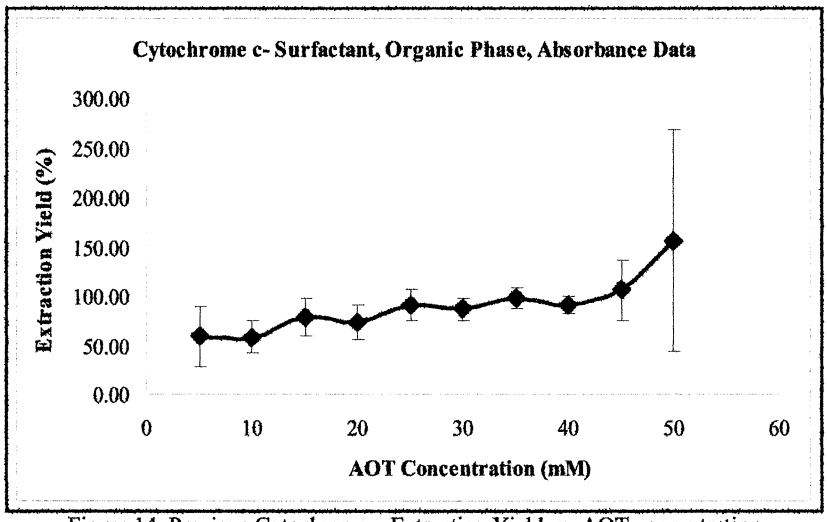

Figure 14. Previous Cytochrome c Extraction Yield vs. AOT concentration (aqueous: 1g/L cyt c, 0.02M Tris buffer, pH~9.7, organic: 5 to 50mM AOT in isooctane)

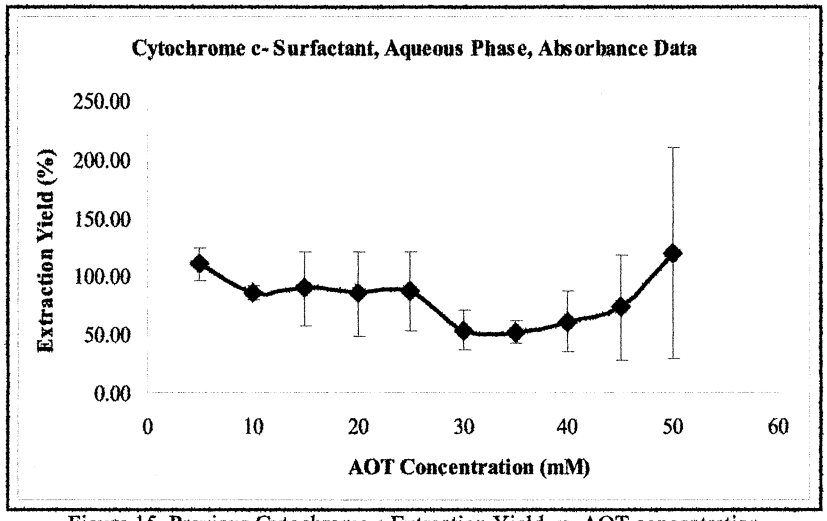

Figure 15. Previous Cytochrome c Extraction Yield vs. AOT concentration (aqueous: 1g/L cyt c, 0.02M Tris buffer, pH~9.7, organic: 5 to 50mM AOT in isooctane)

Table 12. Previous Cytochrome c Surfactant Experiment Data, Organic Phase

AOT	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	St. Dev.
(mM)	A410	Yield %	A410	Yield %	A410	Yield %	A410	(M)	(g/L)	Yield %	
initial	0.57	n/a	0.556	n/a	0.553	n/a	0.56	5.28E-06	0.065	n/a	n/a
5	0.445	78.08	0.129	23.14	0.417	75.34	0.33	3.12E-06	0.039	59	30.96
10	0.414	72.59	0.227	40.9	0.341	61.67	0.327	3.09E-06	0.038	58.51	16.09
15	0.573	100.52	0.361	65.03	0.395	71.39	0.443	4.18E-06	0.052	79.18	18.92
20	0.531	93.14	0.322	58.01	0.385	69.49	0.413	3.89E-06	0.048	73.73	17.91
25	0.521	91.31	0.412	74.25	0.587	106.13	0.507	4.78E-06	0.059	90.55	15.95
30	0.541	94.83	0.413	74.32	0.51	92.19	0.488	4.60E-06	0.057	87.18	11.16
35	0.541	94.84	0.495	89.11	0.613	110.79	0.55	5.19E-06	0.064	98.2	11.23
40	0.565	99.01	0.454	81.66	0.507	91.53	0.508	4.80E-06	0.059	90.81	8.7
45	0.578	101.43	0.436	78.47	0.772	139.58	0.596	5.62E-06	0.07	106.4	30.87
50	1.621	284.18	0.455	81.86	0.552	99.8	0.876	8.26E-06	0.102	156.48	111.99

Table 13. Previous Cytochrome c Surfactant Experiment Data, Aqueous Phase

AOT	Run #1		Run#2		Run#3		Average	Conc.	Conc.	Avg.	St. Dev.
(mM)	A410	Yield %	A410	Yield %	A410	Yield %	A410	(M)	(g/L)	Yield %	
initial	0.57	n/a	0.556	n/a	0.553	n/a	0.56	5.28E-06	0.065	n/a	n/a
5	0.704	123.53	0.622	111.93	0.531	95.99	0.619	5.84E-06	0.072	110.62	13.82
10	0.525	92.02	0.465	83.73	0.445	80.34	0.478	4.51E-06	0.056	85.43	6.01
15	0.534	93.58	0.312	56.2	0.657	118.78	0.501	4.73E-06	0.059	89.52	31.49
20	0.719	126.04	0.313	56.39	0.397	71.71	0.476	4.49E-06	0.056	85.09	36.6
25	0.54	94.66	0.275	49.49	0.642	116	0.486	4.58E-06	0.057	86.75	33.96
30	0.418	73.25	0.227	40.88	0.259	46.8	0.301	2.84E-06	0.035	53.82	17.24
35	0.351	61.46	0.235	42.32	0.285	51.56	0.29	2.74E-06	0.034	51.87	9.57
40	0.222	38.87	0.496	89.24	0.309	55.86	0.342	3.23E-06	0.04	61.13	25.62
45	0.337	59.12	0.205	36.95	0.69	124.71	0.411	3.88E-06	0.048	73.4	45.64
50	1.159	203.27	0.133	23.87	0.723	130.56	0.671	6.33E-06	0.078	119.96	90.24

Cytochrome c Surfactant Experiment- Bioanalyzer Data

For a second method of comparison, both the organic and aqueous phases of the cytochrome c surfactant experiment were analyzed by the Agilent Bioanalyzer 2100. Figure 16 below shows an example of electrophoresis gels of the aqueous phases of one run of one experiment. The leftmost gel, labeled 'L', is the ladder. Gels 1 through 10 are aqueous samples 1 through 10 for a particular run. The lower marker is 4.5kDa and the upper marker is 240kDa. As in a normal gel, a line represents the approximate size of a protein in the sample.

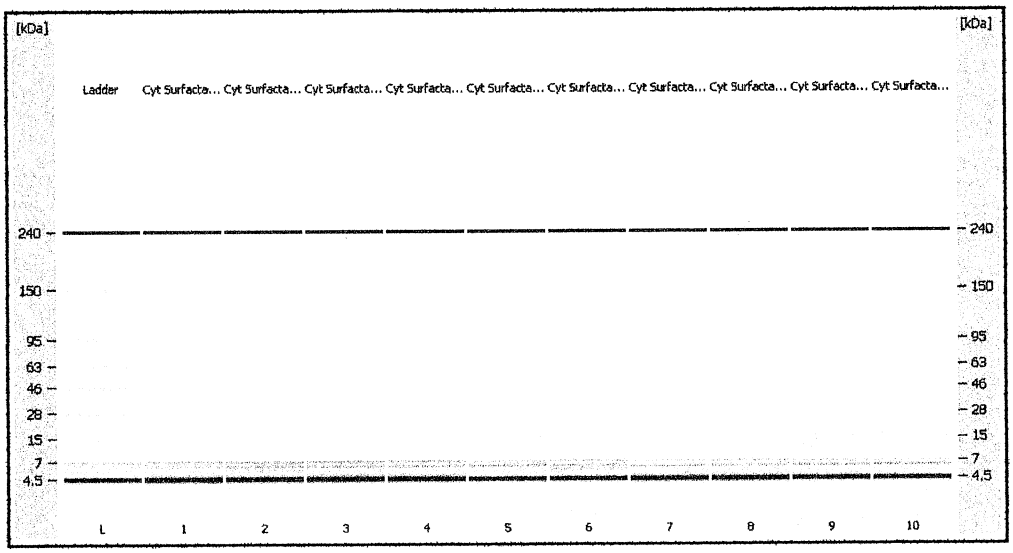

Figure 16. Sample Gel Output of Aqueous phases for Cytochrome c Surfactant Experiment using Agilent Bioanalyzer 2100

Figure 17 below represents a sample electropherogram for the same samples in the figure above. The x-axis is in kDa and the y-axis is in FU (functional units). The Agilent Bioanalyzer quantifies the electropherogram results by calculating the area under the curve. A relative concentration (in ng/uL) and % total is found for each peak on the

electropherogram and corresponding line on the gel, representing a protein of a particular size (kDa). Sample peak tables can be found in Table 14.

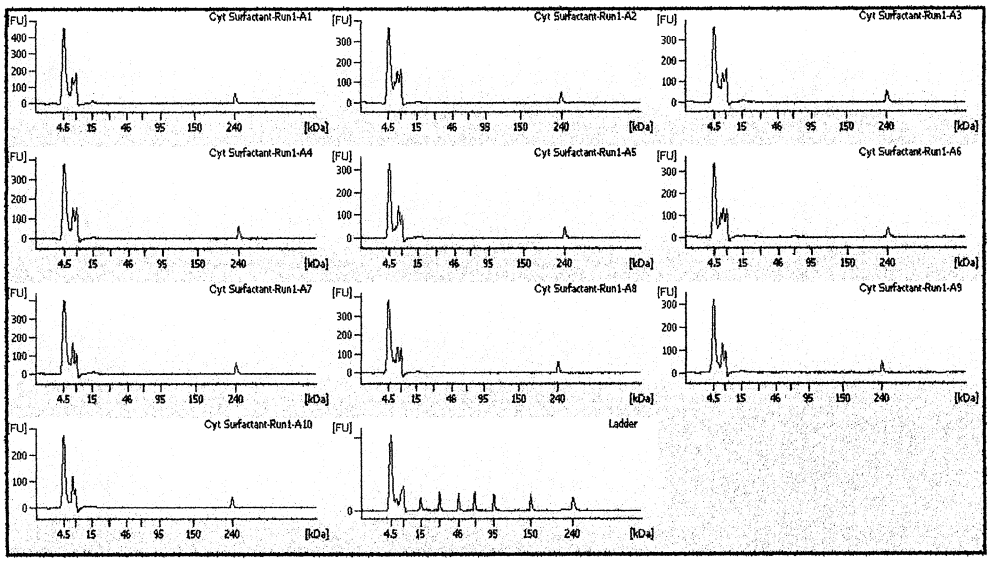

Figure 17. Sample Electropherogram of Aqueous phases for Cytochrome c Surfactant Experiment using Agilent Bioanalyzer 2100

Table 14. Sample Peak Tables for Aqueous phases for Cytochrome c Surfactant Experiment using Agilent Bioanalyzer 2100

Sample	*Peak*	*Size (kDa)*	*Relative Concentration (ng/ul)*	*% Total*	*Observations*
Run 1- Aqueous Sample 1	1	4.5	0.0	0.0	Lower Marker
	2	6.3	0.0	0.0	System Peak
	3	7.1	0.0	0.0	System Peak
	4	15.4	33.3	100.0	-
	5	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 2	1	4.5	0.0	0.0	Lower Marker
	2	5.8	0.0	0.0	System Peak
	3	6.3	0.0	0.0	System Peak
	4	7.0	0.0	0.0	System Peak
	5	15.5	24.2	100.0	-
	6	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 3	1	4.5	0.0	0.0	Lower Marker
	2	6.4	0.0	0.0	System Peak
	3	7.1	0.0	0.0	System Peak
	4	15.4	30.6	100.0	-
	5	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 4	1	4.5	0.0	0.0	Lower Marker
	2	6.4	0.0	0.0	System Peak
	3	7.2	0.0	0.0	System Peak
	4	15.6	14.7	90.4	-
	5	233.7	1.6	9.6	-
	6	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 5	1	4.5	0.0	0.0	Lower Marker
	2	5.8	0.0	0.0	System Peak
	3	6.4	0.0	0.0	System Peak
	4	7.0	0.0	0.0	System Peak
	5	15.4	15.9	100	-
	6	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 6	1	4.5	0.0	0.0	Lower Marker
	2	5.9	0.0	0.0	System Peak
	3	7.2	0.0	0.0	System Peak
	4	15.4	16.8	100	-
	5	240.0	60.0	0.0	Upper Marker
	6	364.9	0.0	0.0	-
Run 1- Aqueous Sample 7	1	4.5	0.0	0.0	Lower Marker
	2	6.3	0.0	0.0	System Peak
	3	7.1	0.0	0.0	System Peak
	4	15.7	27.8	100	-
	5	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 8	1	3.1	0.0	0.0	Lower Marker
	2	4.5	0.0	0.0	System Peak
	3	6.3	0.0	0.0	System Peak
	4	7.2	0.0	0.0	System Peak
	5	16.0	35.4	89.9	-
	6	210.6	1.6	4.2	-
	7	233.5	2.3	5.9	-
	8	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 9	1	4.5	0.0	0.0	Lower Marker
	2	6.4	0.0	0.0	System Peak
	3	7.0	0.0	0.0	System Peak
	4	16.3	19.3	100	-
	5	240.0	60.0	0.0	Upper Marker
Run 1- Aqueous Sample 10	1	4.5	0.0	0.0	Lower Marker
	2	6.4	0.0	0.0	System Peak
	3	16.4	16.8	76.9	-
	4	226.7	2.8	12.7	-
	5	233.3	2.3	10.4	-
	6	240.0	60.0	0.0	Upper Marker

The information in Table 14 can be used to make an extraction profile to compare to Figures 18 and 19. Figures 18 and 19 below show the extraction profiles for cytochrome c varying surfactant concentration using Bioanalyzer results from the organic and aqueous phases, respectively.

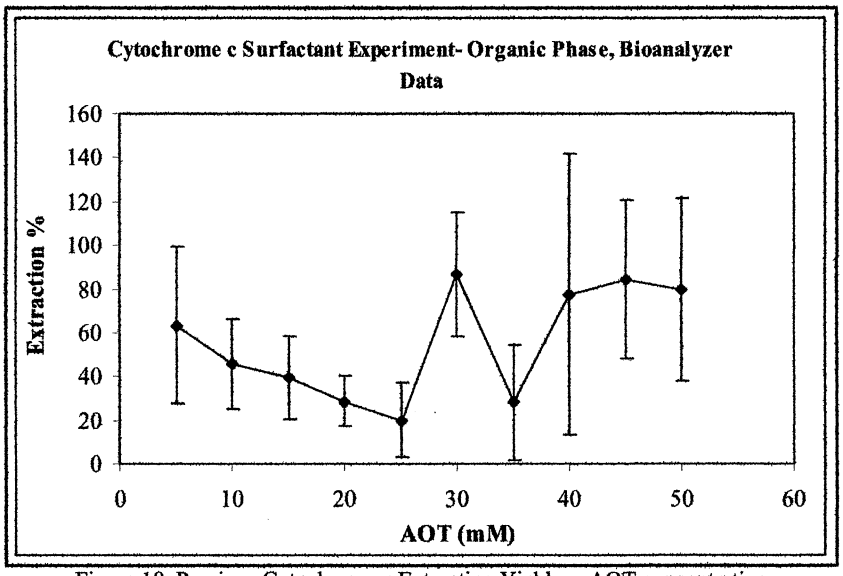

Figure 18. Previous Cytochrome c Extraction Yield vs. AOT concentration (aqueous: 1g/L cyt c, 0.02M Tris buffer, pH~9.7, organic: 5 to 50mM AOT in isooctane)

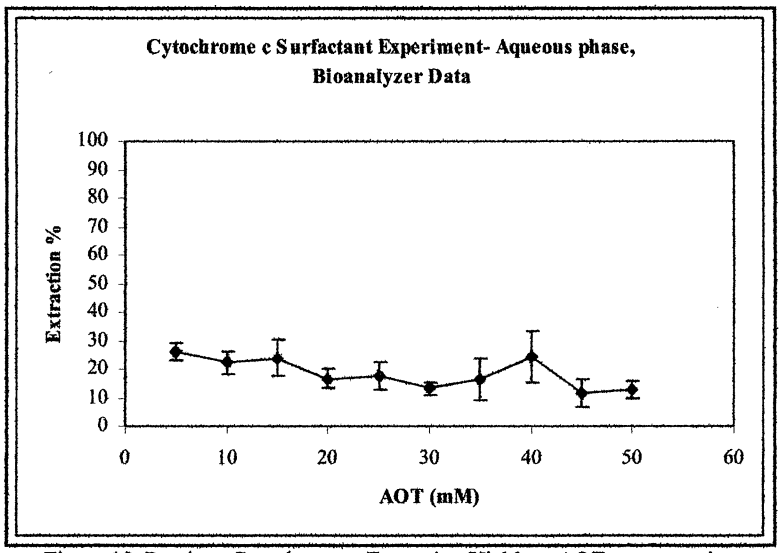

Figure 19. Previous Cytochrome c Extraction Yield vs. AOT concentration (aqueous: 1g/L cyt c, 0.02M Tris buffer, pH~9.7, organic: 5 to 50mM AOT in isooctane)

As with the data analyzed by spectropolarimeter, the organic and aqueous phases analyzed by the Agilent Bioanalyzer do not sum to 100%. Also, Figure 18 does not compare to Figure 14, and Figure 19 does not compare to Figure 15. Therefore, this second method of analysis was not used for the Results section of this thesis.

APPENDIX D

DsRed2 Purification

Examples of anion exchange chromatography graphs for DsRed2 are below. Fractions were collected of the single DsRed2 peak monitored at 561 nm.

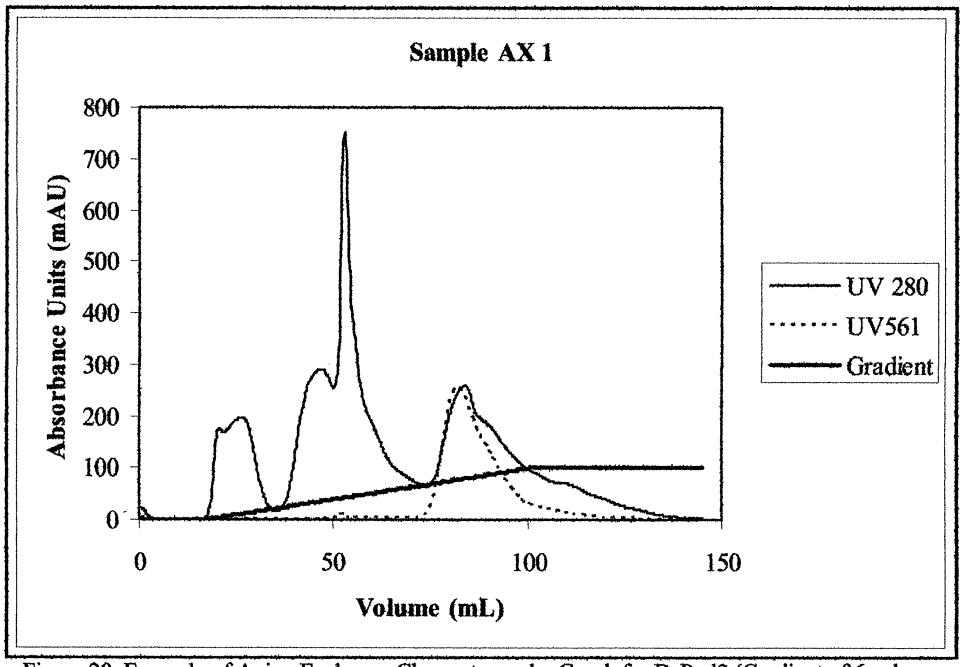

Figure 20. Example of Anion Exchange Chromatography Graph for DsRed2 (Gradient of 6 column volumes)

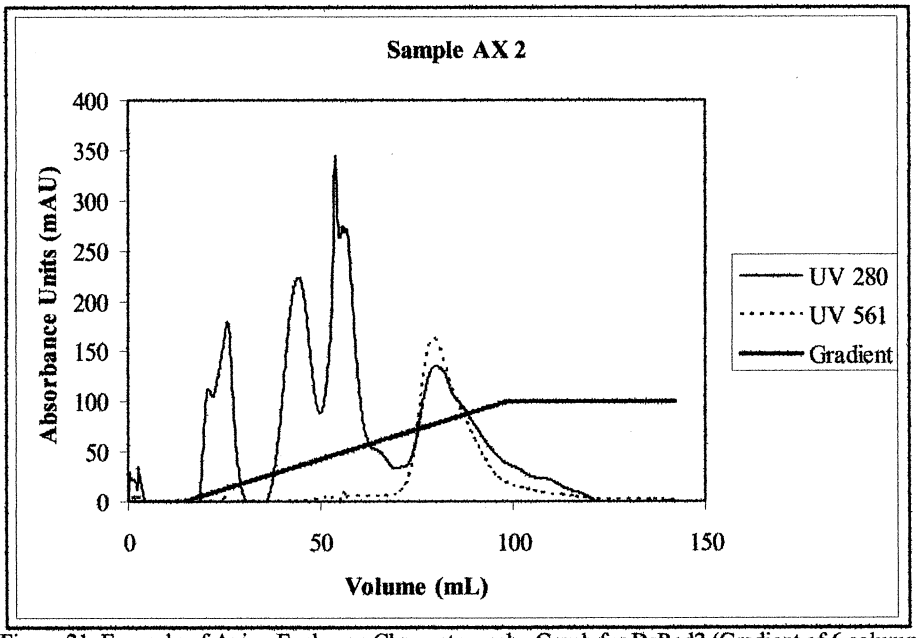

Figure 21. Example of Anion Exchange Chromatography Graph for DsRed2 (Gradient of 6 column volumes)

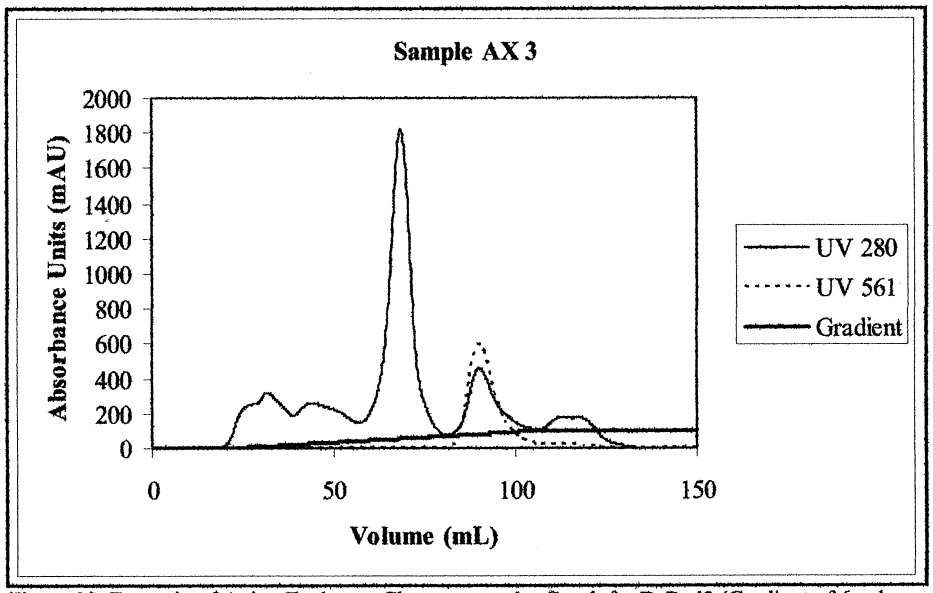

Figure 22. Example of Anion Exchange Chromatography Graph for DsRed2 (Gradient of 6 column volumes)

The purification of DsRed2 after anion exchange chromatography and ultrafiltration was confirmed using the Agilent Bioanalyzer 2100. Examples of the Bioanalyzer output are below.

DsRed2 After AX, Before Ultrafiltration

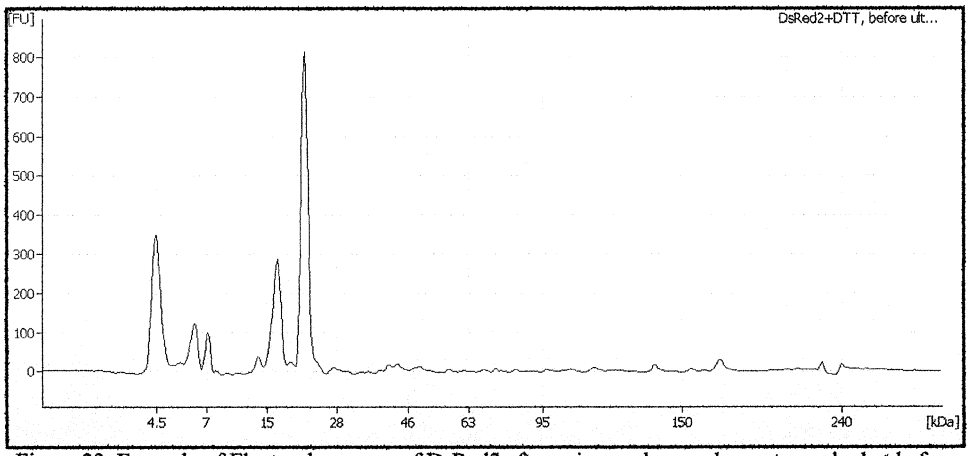

Figure 23. Example of Electropherogram of DsRed2 after anion exchange chromatography but before ultrafiltration

Table 15. Sample Peak Tables of DsRed2 after anion exchange chromatography, but before ultrafiltration

	Size [kDa]	Rel. Conc. [ng/µl]	% Total	Observations
1	4.5	0.0	0.0	Lower Marker
2	5.7	0.0	0.0	System Peak
3	6.4	0.0	0.0	System Peak
4	7.2	0.0	0.0	System Peak
5	13.8	598.8	2.1	
6	17.0	7,794.9	27.9	
7	19.3	368.8	1.3	
8	22.0	17,427.8	62.4	
9	27.4	116.0	0.4	
10	41.3	192.6	0.7	
11	43.3	274.7	1.0	
12	49.5	205.4	0.7	
13	57.6	19.6	0.1	
14	70.0	18.9	0.1	
15	74.8	31.0	0.1	
16	83.5	17.5	0.1	
17	96.8	23.9	0.1	
18	106.0	45.7	0.2	
19	115.3	91.3	0.3	
20	139.5	192.1	0.7	
21	155.7	44.8	0.2	
22	171.3	375.9	1.3	
23	215.0	16.6	0.1	
24	228.8	80.2	0.2	
25	240.0	60.0	0.0	Upper Marker

DsRed2 After AX and UltraFiltration

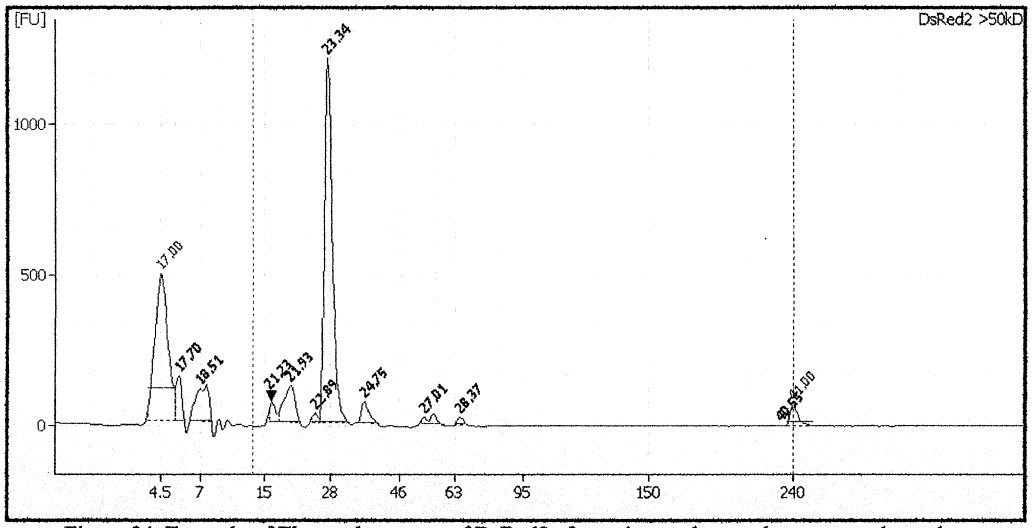

Figure 24. Example of Electropherogram of DsRed2 after anion exchange chromatography and ultrafiltration (50kDa membrane)

Table 16. Sample Peak Tables of DsRed2 after anion exchange chromatography and ultrafiltration (50kDa membrane)

	Size [kDa]	Rel. Conc. [ng/µl]	% Total	Observations
1	4.5	0.0	0.0	Lower Marker
2	5.6	0.0	0.0	System Peak
3	7.0	0.0	0.0	System Peak
4	16.5	72.8	3.3	
5	20.1	255.2	11.4	
6	25.1	26.9	1.2	
7	27.4	1,724.1	77.2	
8	37.0	90.9	4.1	
9	53.7	44.4	2.0	
10	65.6	18.2	0.8	
11	232.6	1.1	0.0	
12	240.0	60.0	0.0	Upper Marker