ABSTRACT

Title of Thesis:	DISSOLVED PROTEIN MODIFICATION AND DEGRADATION IN NATURAL WATERS
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Organic nitrogen is an important part of the nitrogen cycle in oceans, with both particulate and dissolved forms serving as substrates for bacterial growth. Proteins are the principal organic nitrogen compounds of living biomass and therefore believed to be very labile; however, some portion can be found in dissolved organic matter pools. Experimental incubations were used to examine the structural modification and degradation of the model protein bovine serum albumin (BSA) in different aquatic environments. Size-exclusion chromatography along with amino acid analysis and electrospray ionization mass spectrometry showed that high molecular weight material formed over the time course of the incubation was resistant to degradation. Low molecular weight products were formed from sequential hydrolysis, with the selective removal of polar, charged amino acids and were rapidly utilized by the bacteria present. As degradation of the protein products progressed, the amino acid composition shifted back towards the original protein composition.

DISSOLVED PROTEIN MODIFICATION AND DEGRADATION IN NATURAL WATERS

By

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Chapter 1: Contribution of Protein to the Organic Matter Pool in Aquatic Environments

Primary production by phytoplankton is a primary source of organic matter in marine systems (Suess 1980). This organic pool includes phytoplankton as particles but also significant amounts of dissolved organic matter (DOM) produced during photosynthesis (Bjørsen 1988; Baines and Pace 1991) or subsequent recycling reactions, including direct release by microplankton (Eppley, Horrigan et al. 1981) and sloppy feeding by bacteria (Fuhrman 1987). The major identified components of dissolved organic matter (DOM) and particulate organic matter (POM) include amino acids, carbohydrates, and smaller amounts of lipids (Mannino and Harvey 1999; Mannino and Harvey 2000), yet a significant fraction of DOM remains uncharacterized. Although the nitrogen composition of living phytoplankton is known (Fig. 1.1), the link between the nitrogen produced by phytoplankton or degrading organisms and the refractory pool remains unclear.

Heterotrophic bacteria are major consumers of organic matter, remineralizing at least 50% of all primary production (Pomeroy 1974; Azam, Fenchel et al. 1983). Nevertheless, a small fraction of this organic matter is preserved in aquatic environments. A comprehensive understanding of organic matter sources, sinks and fate is crucial to understanding the relationship between the carbon cycle and anthropogenic change in the environment. Little is known about the detailed composition of organic matter preserved on long time scales.

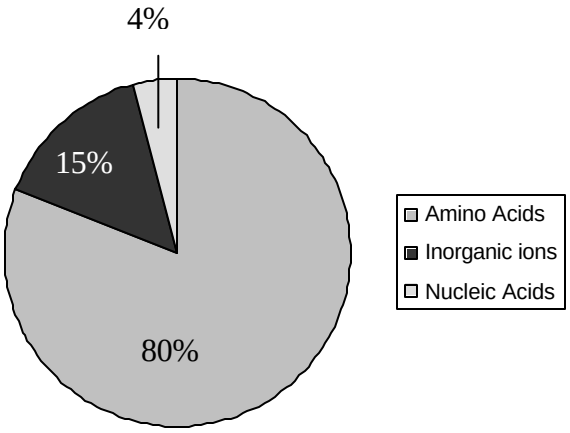


Figure 1.1. The distribution of major nitrogenous components to total nitrogen in marine phytoplankton. Plotted after data from Lourenço et al. (1998).

1.1 Organic Matter Composition in Aquatic Environments

Particulate organic matter (POM), which is operationally defined by size and contains a combination of living organisms and detrital matter, has been extensively studied for decades. It is composed of living cells, as well as living organism fragments, fecal pellets, aggregates of DOM, and DOM that is sorbed to minerals (Little 1985; Mayer 1994; Hedges and Keil 1999; Mannino and Harvey 2000). Surface water samples of POM from a number of marine locations are similar to each other and to live plankton, a major contributor to POM (Tanoue 2000), in terms of size and composition. For example, Tanoue (1985) found 50-80% of particulate organic matter could be identified as amino acids, carbohydrates, and lipid. However, for dissolved materials, generally less

than 30% of DOM has been characterized as amino acids, carbohydrates, and lipids, and this percentage varies by location (Williams and Druffel, 1987; Mannino and Harvey 2000). Bulk properties of ultrafiltered DOM (<0.1um), including C/N ratios, stable isotope ratios, radiocarbon ages, and overall amino acid content are all similar to total DOM, but are often very different from marine organism composition and detrital particles (McCarthy, Hedges et al. 1993; McCarthy, Hedges et al. 1996; Benner, Biddanda et al. 1997). Dissolved organic matter is more difficult to characterize because of low concentrations in a salty mineral matrix, high complexity, and high solubility (Williams and Druffel 1987).

Researchers commonly separate DOM into high and low molecular weight fractions to examine bacterial utilization. Over the years the definition of DOM has changed following filter size changes. Unfortunately, the operational definition of the high and low molecular weight DOM makes it difficult to compare past results. For example, Koike, Hara et al. (1990) and Wells and Goldberg (1991) found that the high molecular weight fraction of DOM (>10,000 Da) was predominant in the upper ocean relative to the low molecular weight fraction of DOM. However, Benner et al. (1992) found low molecular weight material (<1000 Da) to be approximately 65-75% of total DOM throughout the ocean (Ogawa, Yukio et al. 2001). Comparing these two examples, it is unknown what happens to the DOM in the size fraction between 1000 and 10,000 Da.

Even so, it is commonly believed that lower molecular weight DOM is remineralized rapidly in the water column. Supporting this idea is that marine bacteria must breakdown proteins, defined as high molecular weight, into amino acids or small

peptides prior to direct uptake, therefore creating low molecular weight material (Hollibaugh and Azam 1983). Confounding these findings, Williams and Druffel (1987) found the average age of total DOM to be approximately 6,000 years This age suggests that low molecular weight DOM, the bulk of DOM, is actually much older and less reactive than previously believed. Using ^{14}C dating, (Santschi, Guo et al. 1995) found that the high molecular weight fraction of DOM (>1000 Da) is young, more easily characterized, and consists mainly of carbohydrate-like material (Benner, Pakulski et al. 1992). Recent studies have shown that larger molecules are in fact consumed readily by bacteria (Amon and Benner 1996; McCarthy, Hedges et al. 1996). These data led Amon and Benner (1996) to create the size-reactivity continuum model to explain reactivity of DOM (Fig. 1.2). This model suggests that bioreactivity of organic matter decreases along a continuum of size and diagenetic state (Amon and Benner 1994; Amon and Benner 1996). The low molecular weight fraction of DOM is characterized as rich in organic nitrogen and is believed to be generally proteinaceous material; whereas HMW is found to contain more inorganic nitrogen and include more polysaccharides (Amon and Benner 1994).

To clarify past research, it is important to understand microbial processes affecting organic compounds. Attempts have been made to understand this link between living marine organisms and DOM by using biomarkers and bulk chemical characterizations (Tanoue et al. 1996). Such attempts have included measurements on bulk elements (e.g. C, N, S and P) with colorimetric assays, or at the monomer level (e.g. amino acids, fatty acids, and monosaccharides) after hydrolysis. With these approaches, it is difficult to obtain information concerning the source of the material or how living

organisms affect the composition. Improving current biochemical and molecular techniques, such as ultrafiltration, high performance liquid chromatography (HPLC), gas chromatography (GC), and LC/mass spectrometry, will allow for isolation, separation, and examination of molecules in the complex matrix of seawater.

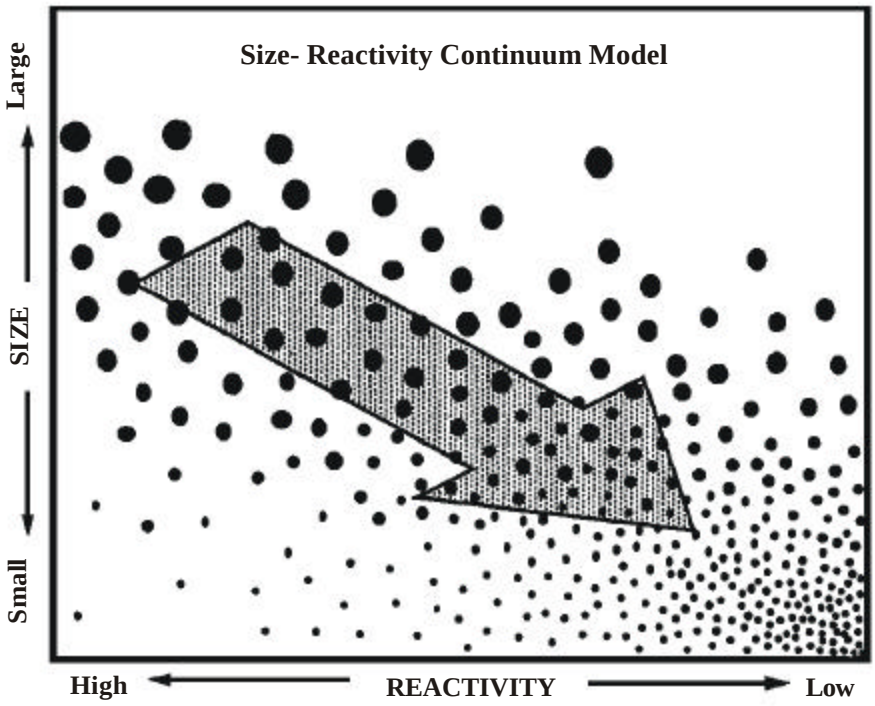
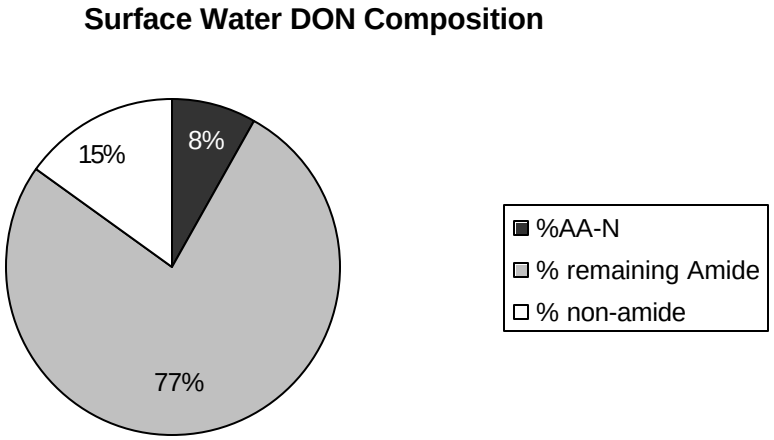


Figure 1.2. Conceptual diagram of the size-reactivity continuum model for organic matter decomposition in marine environments. Arrow shows the major pathway of degradation. The size of the dots represents organic matter size with POM being the larger to low molecular weight DOM being the smallest. Model from Amon and Benner (1996).

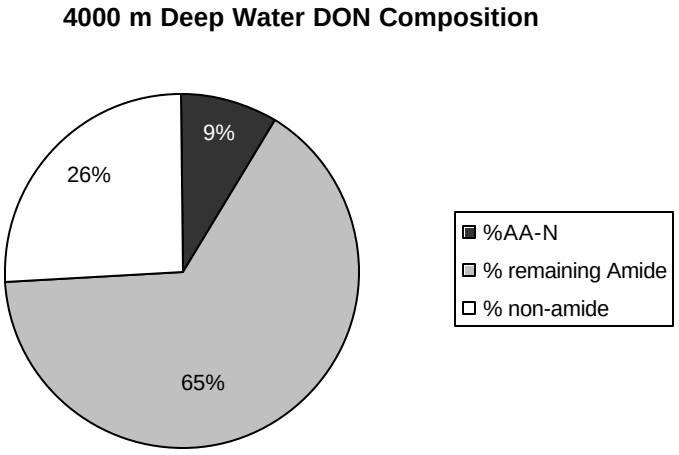
1.2 Possible Contributors to Aquatic Dissolved Organic Nitrogen Pool

A minor fraction of dissolved organic nitrogen (DON) is accounted for by small rapidly recycled organic compounds (Smith, Krohn et al. 1985). Most DON resides in nitrogenous substances that appear to resist degradation (Harvey, Boran et al. 1983). Early work suggested that DON consists of largely complex macromolecules formed

from biological degradation, followed by spontaneous abiotic condensation of biochemical precursors (Harvey, Boran et al. 1983). More recent results from McCarthy, Pratum et al. (1997) showed that most high molecular weight DON exists in an amide form analogous to that seen in peptides, suggesting biological formation (Figure 1.3).



a)



b)

Figure 1.3. Percentages of three broad categories of organic nitrogen forms within ultrafiltered samples from (a) surface water, and (b) deep water in the Pacific Ocean. Differences between ultrafiltered DON and total DON may be heterocyclic nitrogen forms. Data from McCarthy et al. (1997)

There are three main sources of amide linkages in marine systems and include chitin, peptidoglycan, and proteins. Chitin, a polymer of the amino sugar N-acetylglucosamine, is released by the breakdown of bacteria, cyanobacteria, and algal cell walls (Blackwell, Parker et al. 1967; Smucker and Dawson 1986; Poulicek, Gaill et al. 1998). A recent study by Benner and Kaiser (2003) found low concentrations of chitin in POM and ultrafiltered DOM in all samples measured, suggesting chitin is not a main contributor to the DON pool.

Peptidoglycan is a constituent of gram positive bacterial cells and suggested as a important contributors to marine DOM, but little is known about its distribution and turnover. Nagata, Meon et al. (2003) used kinetic studies to compare radiolabelled peptidoglycan and protein remineralization and hydrolysis rates and found that protein is degraded 2-21 times faster than peptidoglycan. The experiment also showed that the radiolabelled peptide portion of peptidoglycan is degraded three times faster than the radiolabelled polysaccharide portion (Nagata et al. 2003). Low molecular weight, chemically modified material was formed during the peptidoglycan degradation, but not during protein degradation (Nagata, Meon et al. 2003). Benner and Kaiser (2003) found muramic acid, an amino acid found only in bacterial cell wall peptidoglycan, in high concentrations in POM, but found it to be a minor component of ultrafiltered DOM. Given that 60-80% of nitrogen in living biomass is present as hydrolysable amino acids (Cowie and Hedges 1992), and that proteins are the most abundant nitrogen-containing substances in phytoplankton (Brown 1991; Lourenço, Barbarino et al. 1998), it can be hypothesized that a majority of the dissolved organic nitrogen pool with amide linkages

originates as proteinaceous material. However, this protein has undergone modifications so that it cannot be easily hydrolyzed.

1.3 Protein as a Contributor to the Aquatic Dissolved Nitrogen Pool

Proteins are large molecules that contain one or more polypeptides stabilized by interactions including as covalent disulfide bonds and hydrogen bonding, van der Waals, or hydrophobic interactions. There is a wide distribution of protein sizes, ranging from 50 to 25,000 amino acid residues (Creighton 1993). It is important to note that about 50% of naturally found proteins are glycosylated. Algae contain ribulose bisphosphate carboxylase/oxygenase (RUBISCO), which is the most abundant protein in the world (Creighton 1993).

Proteins have highly variable chemical characteristics with respect to structure, hydrophobicity, and function. Intracellular proteins are soluble in seawater, whereas membrane proteins are less soluble and usually contain more hydrophobic amino acids than other proteins (Creighton 1993). Proteins in seawater are found in a number of physical states: denatured, native, solute, sorbed, and complexed with DOM and minerals (McConville, Fisher et al. 1982; Keil and Kirchman 1993; Keil and Kirchman 1994; Taylor, Troy et al. 1994; Poulicek, Gaill et al. 1998).

The possible sources of dissolved proteins found in organic matter have been of substantial interest in recent years; however, there are no simple methods to date for investigating the chemical nature of proteins in aquatic environments. Tanoue (1996) used sodium dodecyl sulfate-polyacrylamide gel electrophoresis to investigate the size

distribution of dissolved protein in the marine environment. Results showed that a small number of protein species (<30) contributed to the remaining dissolved organic nitrogen pool (Tanoue, Nishiyama et al. 1995).

Dissolved proteins greatly differ from particulate proteins in that the particulate fraction is made up of a large number of different proteins all present in low levels (Tanoue, Ishii et al. 1996). Particulate protein was found to be correlated with chlorophyll and particulate organic carbon, and accounts for 12-32% of particulate nitrogen in surface and deep waters (Packard and Dortch 1975; Tanoue 1992). These data are in agreement with the hypothesis that either proteins produced from primary production are not transferred to the dissolved phase or they are biologically used rapidly

As important sources of both carbon and nitrogen, proteins are considered attractive as substrates for bacterial growth and therefore are typically recycled rapidly in the water column (Hollibaugh and Azam 1983). Bacterial uptake rates have been measured in a number of kinetic studies (Hollibaugh and Azam 1983; Keil and Kirchman 1991b; Panjota and Lee 1999). Dissolved protein degradation rates can be as fast as amino acid degradation in seawater (Hollibaugh and Azam 1983). In experiments with pure proteins, most cellular proteins were found to be utilized within hours to days (Hollibaugh and Azam 1983; Keil and Kirchman 1993); however, these experiments do not mimic the complexity of organic matter found in the water column.

Bacteria must breakdown proteins into amino acids or small peptides prior to direct uptake. Two theories on bacterial uptake of protein involve breakdown a) within b) or outside the cell (Hollibaugh and Azam 1983). The breakdown of proteins to peptides outside the cell is the prevailing theory, because peptides are commonly found in

the environment, but this has yet to be adequately tested (Hollibaugh and Azam 1983). In agreement with this theory, Hollibaugh and Azam (1983) found that larger peptides were rapidly turned over and there was a shift to smaller peptides (<700 Da) during protein degradation without intermediate sizes being observed. These data suggest that bacterial enzymes have an affinity first for intact proteins, then large peptides, followed by small peptides (Hollibaugh and Azam 1983).

There is conflicting evidence that intermediate materials of smaller sizes originate from proteins and peptides. Keil and Kirchman (1991) and Pantoja and Lee (1999) suggested that degradation of proteins is a step-wise process and the rate of hydrolysis depends on the sizes of the peptides formed. Bacteria might release extracellular enzymes into the environment to hydrolyze proteinaceous material, but this seems energetically unfeasible because of diffusion of the enzymes away from the cell and consequently diffusion of the peptides formed into surrounding environments (Mayer, Schick et al. 1995; Vetter, Jumars et al. 1998). Regardless of these observations, different bacteria favor different substrates (Cottrell and Kirchman 2000), so to simplify protein degradation in all aquatic environments to a single process may not be possible. Although other organisms may also degrade dissolved proteins, utilization by heterotrophic flagellates is the only other organism examined to date (Tranvik, Sherr et al. 1993).

1.4 Possible Pathways of Dissolved Protein Preservation

Proteinaceous material is preserved under certain circumstances. Evidence can be found in examinations showing the presence of amino acids in fossil shells (Abelson 1954), selected proteins in coastal and ocean waters in the Pacific Ocean (Siezen and Mague 1978; Lee and Cronin 1984), within sediments as old as 4000 years (Nguyen and Harvey 2001) and even encapsulated in organic matrices of 4 million year old kerogen (Nguyen and Harvey 2003). Theories on protein preservation pathways (Fig. 1.4) include selective preservation of inherently refractory molecules, abiotic condensation reactions (Ishiwatari 1992), sorption to mineral surfaces (Keil and Kirchman 1994), and encapsulation within a protective layer (Knicker and Hatcher 1997). Selective attack on specific protein amino acids is unlikely, because total amino acid composition appears to be very similar among aquatic environments including the Delaware estuary (Keil and Kirchman 1993), coastal regions (Siezen and Mague 1978), and at various depths in the open ocean (Wakeham et al. 1994; Panjota and Lee 1999). Further investigation is necessary to fully understand the contribution of selective degradation to protein preservation.

As possible evidence of selective preservation, membrane proteins are known to have lower proteolysis rates in the water column than soluble proteins (Laursen, Mayer et al. 1996; Nagata, Fukuda et al. 1998). It is important to note that within membranes, proteins will likely be degraded by ectoenzymes of bacteria (Martinez, Smith et al. 1996). Membrane proteins are not necessarily believed to be less resistant to degradation based on structure, but because they complex with macromolecules, steric hindrances affect

their hydrophobic nature (Nagata, Fukuda et al. 1998). If membrane proteins are degraded more slowly, there is more time for modification of protein into refractory material.

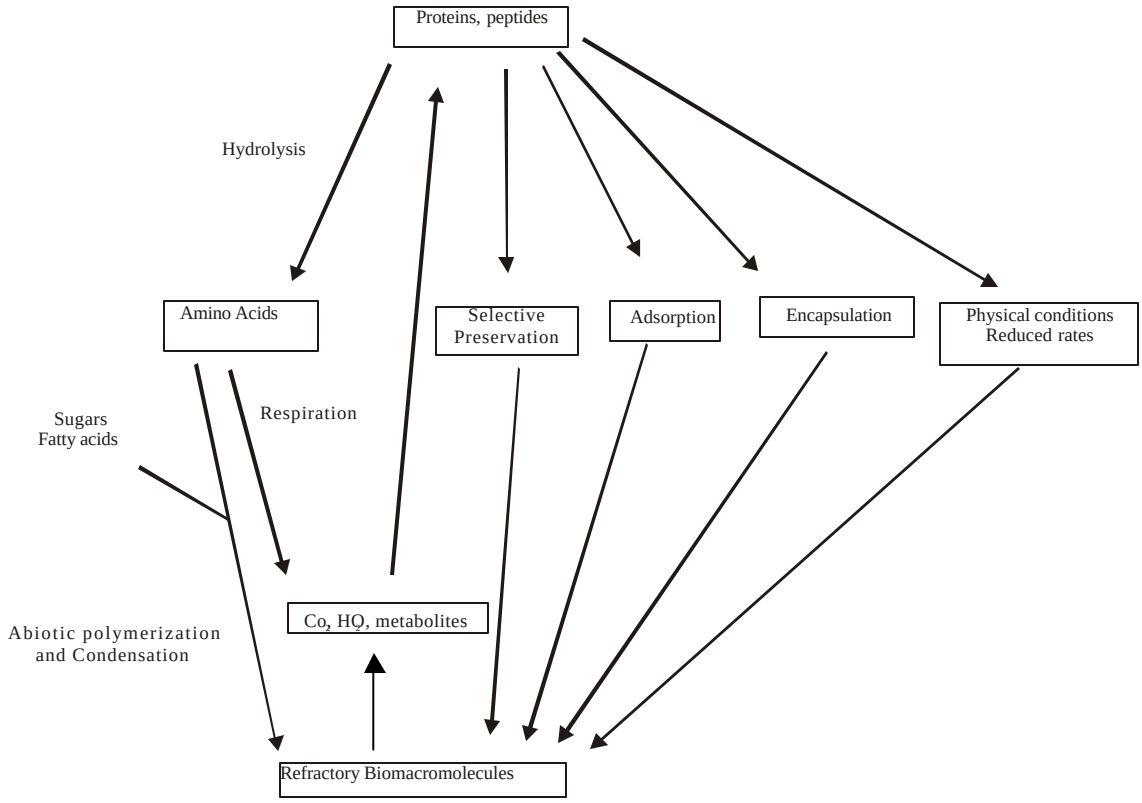


Figure 1.4. Scheme illustrating current views on the fate of proteins in aquatic systems. Several preservation pathways of dissolved proteins exist. Protein can be further broken down, altered and added to the refractory pool. The rest is remineralized by bacteria and returned to active biogeochemical cycles.

Abiotic transformations, including condensation, adsorption, and photochemical reactions, have the potential to act as preservation pathways (Hedges, Clark et al. 1988; Keil and Kirchman 1994; Nagata and Kirchman 1996). One mechanism might include the spontaneous deamidation of asparagine and glutamine (Creighton 1993) which

converts them to isoaspartic acid, thereby changing the peptide location and the structure of the protein. This structural change could have a significant effect on protein degradability. Another preservation possibility is dissolved protein complexation, such as glycosylation, which may be common and may render proteins resistant to biological degradation (Keil and Kirchman 1993; Keil and Kirchman 1994). Lastly, Otte, Lomholt et al. (1997) established that limited protein hydrolysis can lead to protein aggregation, presumably due to structural unfolding during hydrolysis which might also aid preservation.

Refractory DOM could include integral membrane proteins only partially soluble in seawater, which causes aggregation (Nguyen 2000). Porins, which form trans-outer membranes and water filled channels on the outer membranes of gram negative bacteria, have been suggested to be selectively preserved (Tanoue 2000). Tanoue (1995) examined the distribution of protein molecular weights found in DOM using ultrafiltration, TCA precipitation, and gel electrophoresis. A 48 kDa protein was discovered in the dissolved phase, and, using a protein database, was found to match porin P (Tanoue, Nishiyama et al. 1995). The mechanisms of degradation and preservation appear myriad but similar in all marine environments examined.

Adsorption of proteins and its effect on protein degradation has been studied over recent years. In laboratory studies, proteins adsorbed to glass beads are more slowly degraded than freely dissolved proteins (Nagata and Kirchman 1996). Hydrolysis rates were not influenced by collision of bacteria to sorbed protein, but instead were affected by different protease affinities for protein (Nagata and Kirchman 1996). There is evidence that adsorption may temporarily store labile DOM in slowly degrading, semi-

labile pools making it available for export from the water column (Nagata and Kirchman 1996).

Taylor (1995) measured the effects of protein concentration and adsorption rates on degradation by marine bacteria. Results showed that sorbed proteins are degraded more rapidly than dissolved proteins at the same concentrations (Taylor 1995). Although this finding contradicts other experiments, which show slower degradation rates for adsorbed proteins (Samuelsson and Kirchman 1990; Mayer 1994; Nagata and Kirchman 1996), Taylor (1995) observed that the type of protein and substrate can be important factors in sorption of protein. Adsorbed proteins may actually be degraded more rapidly due to concentration and subsequent denaturation between the substratum and internal hydrophobic surfaces, exposing an increased number of bonds for hydrolysis (Soderquist and Walton 1980; Taylor 1995).

Encapsulation of protein can occur through interaction of aliphatic amino acid residues with paraffinic regions of humic acids (Zang, van Heemst et al. 2000) or via aggregated DOM and particles trapping protein within a liposome-like enclosure (Borch and Kirchman 1999). Membranes of gram-negative bacteria and cell walls have phospholipid, peptidoglycan and lipopolysaccharides that can complex with membrane proteins by hydrophobic interactions and these membrane fragments can form liposomes. Encapsulation greatly decreases protein degradation rates (Borch and Kirchman 1999), suggesting that labile proteins can be physically protected from degradation by encapsulation, allowing them to remain in the DOM pool for longer periods of time, making them available for modification.

1.5 Total Hydrolyzable Amino Acids Approach to Protein Degradation Studies

Researchers have commonly measured amino acids after acid hydrolysis of peptide bonds because of the difficulties in working with dissolved proteins. Many studies have measured total hydrolysable amino acids, combined amino acids in both the dissolved and particulate phases, and free amino acids (Siezen and Mague 1978; Lee and Cronin 1984; Cowie and Hedges 1992). Studies have shown that many marine organisms have similar amino acid composition and major amino acid groups have similar rates of degradation (Fig. 1.5), although Nguyen and Harvey (1997) found small increases in serine and glycine during degradation. Neutral amino acids are the most abundant group in seawater, followed by acidic, aromatic, and lastly basic amino acid groups (Nguyen and Harvey 1997). Hecky, Mopper et al. (1973) found similar results within a diatom

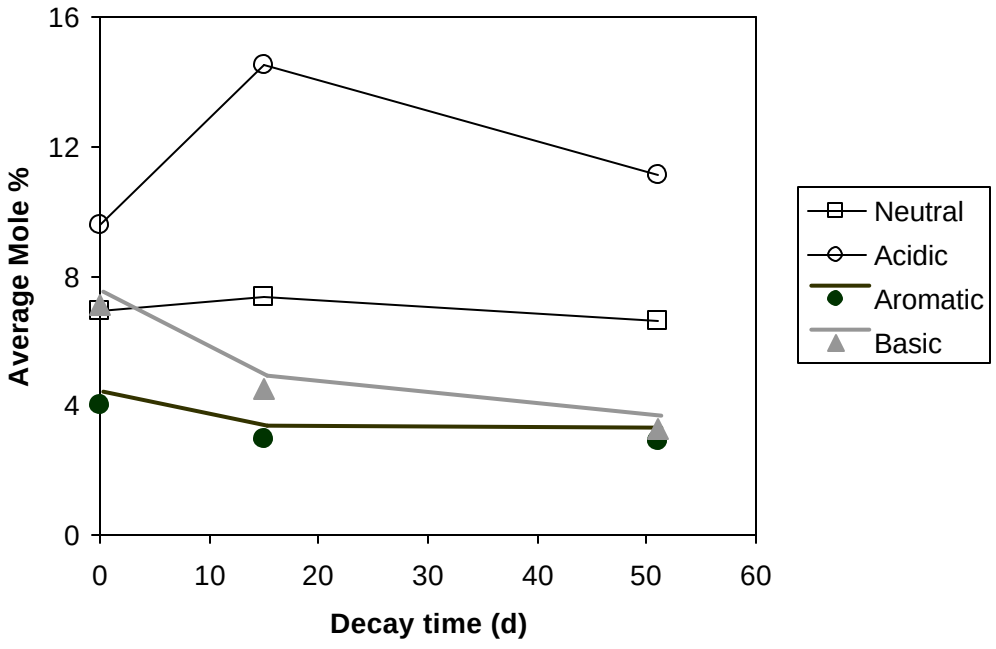


Figure 1.5. Time course of the average mole percent shifts in particulate amino acid carbon during the oxic decay of diatom, *Thalassiosira weissflogii*. Data from Nguyen and Harvey (1997).

cell wall being enriched in neutral amino acids (e.g. serine, glycine and threonine) and depleted in aromatic, acidic, and sulfur-containing amino acids. However, it is unknown what happens to proteins once they are released into the environment. An important factor affecting the accuracy of amino acid analyses of DOM is the hydrolysis method used. Keil and Kirchman (1991) investigated the efficiency of the traditional acid hydrolysis method (6N HCl) on DOM and found that 6N HCl greatly underestimates dissolved combined amino acids in seawater because of the lack of complete hydrolysis within the complex organic matter matrix. The vapor-phase hydrolysis method was found to more completely hydrolyze proteinaceous material within DOM. This finding could mean that DOM is more similar in composition to organisms than previously believed, but due to alterations or modifications resulting in lowered hydrolysis, much of the amino acid concentrations are significantly underestimated.

Dissolved combined amino acids (including peptides and proteins) and dissolved free amino acids are believed to be released from phytoplankton directly (Myklestad, Holm-Hansen et al. 1989), by viral lysis and autolysis of dead algae (Gardner, Chandler et al. 1987), and by protists grazing on bacteria increasing the concentrations of dissolved combined amino acids (Ferrier-Pages, Karner et al. 1998). Dissolved free amino acids are believed to be utilized more rapidly as a source of carbon, nitrogen, and energy (Williams, Berman et al. 1976) and usually remain lower in concentration than the combined forms, as in peptides and proteins in the water column (Keil and Kirchman 1991; Keil and Kirchman 1991). Coffin (1989) shows differences in individual amino acid abundances (Fig. 1.6) from dissolved free amino acids (high in glycine, serine,

glutamic acid, and aspartic acid) to dissolved combined amino acids (high in valine, phenylalanine, isoleucine, and leucine). These differences may indicate that there may be a preferential utilization of dissolved combined amino acids because they contain a more diverse array of amino acids for bacteria to utilize than dissolved free amino acids. On

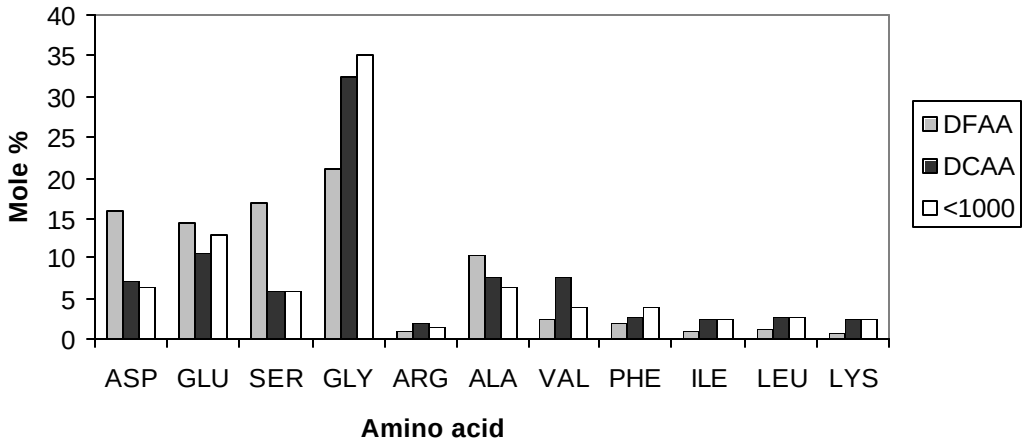


Figure 1.6. Analysis of individual amino acids in the dissolved free amino acid fraction (DFAA), dissolved combined amino acid fraction (DCAA), and the <1000 Da fraction from the Delaware Estuary as a comparison of individual amino acid composition differences among them. Redrawn from (Coffin 1989).

the other hand, certain dissolved free amino acids may be preferentially hydrolyzed over others, meaning that some amino acids are utilized more rapidly than others causing a skewing of the distribution within the pool. Phytoplankton can use dissolved free amino acids possibly by cell surface amino-oxidases which hydrolyze amino acids to ammonia (Pantoja and Lee 1994). Dissolved combined amino acids are found to be anywhere from 20 to 100% more abundant than the free forms, but used by bacteria 2-10 times more slowly (Azam, Smith et al. 1992; Keil and Kirchman 1993). Because amino acids have been extensively studied, whole protein characterization coupled with amino acid

composition analysis would give a clearer picture of dissolved protein degradation in aquatic systems.

1.6 Research Objectives

A major objective of this thesis is to examine bacterial degradation of protein and modification intermediates formed over time. Following the size-reactivity continuum presented by Amon and Benner (1996), I hypothesized that as bacteria use protein, the molecular weight decreases and the degradation products will be less bioreactive, and will be preserved for a longer period of time. Another possibility is that some portions of the protein products will be selectively preserved by aggregation of peptides produced or by a combination of abiotic condensation and bacterial modification. All these processes make them less available for utilization. Bacterial incubations in the laboratory were performed to examine relative rates of protein degradation and changes in amino acid composition over a short incubation time. Techniques commonly used for quantification, separation, and determination of proteins were modified for use in the complex matrix of seawater. The major hypotheses were as follows: (1) bacteria will readily use protein as a substrate, and as a result, a shift toward smaller molecular weight degradation products, facilitated by bacterial proteases; (2) shifts in amino acid composition of modified products will occur because some amino acids are more available to bacteria due to protein structure; (3) a small fraction of protein will shift to multiple lower molecular weight products as hydrolytic intermediates are formed whereas the largest fraction will be remineralized. Because tracking protein within a complex matrix of organic matter is difficult, I followed a model protein, BSA, added to natural water incubations. The

physical and chemical characteristics of BSA are well known, it is readily available, and has been used in other experiments, which provides a thorough examination of dissolved protein degradation in natural waters.

Chapter 2: Method Development for the Analysis of Dissolved Proteins in Estuarine and Marine Samples

2.1 Introduction

The nitrogen cycle plays an important role in aquatic systems and the characterization and contribution of dissolved nitrogenous matter is important to the cycling of organic matter. Dissolved combined amino acids are the largest identifiable fraction of dissolved organic nitrogen in aquatic systems, and an important source of nutrition for bacterial growth (Coffin 1989; Keil and Kirchman 1991). As reviewed in Chapter 1, a number of efforts have been made to quantify and characterize dissolved proteins using a variety of methods developed to measure proteins in aquatic systems.

There are several major obstacles in the examination of dissolved organic materials in natural waters. These include 1) the difficulty in separating all phases of organic matter from the inorganic salt matrix in which they are dissolved and 2) the inability to easily separate organic matter into similar compound groups for characterization (Leenheer 1981). More specifically, substances such as dissolved proteins are difficult to quantify due to their apparent low concentrations in the aquatic environment (Tanoue 1995), the complexity and heterogeneity of naturally occurring proteins which might occur (Hollibaugh and Azam 1983; Tanoue 1995), and the difficulty of separating dissolved proteins from humics, minerals, and other compounds in seawater that interfere with common protein measurements (Lowry, Rosebrough et al. 1951; Smith, Krohn et al. 1985). Proteins are complex structures, and are typically found

in a variety of different forms. Consequently, generic protocols must be applicable to proteins as a whole. Proteins can differ in hydrophobicity, shape, size, solubility, and likelihood of aggregation. However, during extraction and quantification, the distinctive properties of individual proteins are not likely as significant as the matrix the protein is in. Existing methods require sample pretreatment to remove interfering substances present in natural waters.

In order to develop methods to quantify and characterize proteins, as well as examine the degradation processes that occur in natural waters, a model protein was used. Bovine serum albumin (BSA) was chosen because it is well characterized, and readily available in high purity. It has been used as a model protein in other natural water experiments (Hollibaugh and Azam 1983; Kirchman, Henry et al. 1989; Nguyen and Harvey 1994) and is therefore appropriate for comparison of results. In the following sections, a brief description of past methods for the extraction, concentration, and quantification of protein is provided including the advantages and disadvantages of each method. The testing of methods is then described, followed by a description of the methods chosen.

2.1.1 Extraction and Concentration

Detailed structural analysis using methods such as nuclear magnetic resonance (NMR), infrared spectrometry, and liquid chromatography/ mass spectrometry (LC/MS), requires that salts and other interfering compounds found in aquatic systems be removed. Historically hydrophobic macroreticular (XAD) resin (Leenheer 1981; Thurman and

Malcolm 1981; Amador, Milne et al. 1990), freeze-drying, trichloroacetic acid precipitation, C-18 cartridges, and ultrafiltration have been used to extract and concentrate organic matter from aquatic systems. XAD resins were found to only capture 15% of all organic matter (Benner, Pakulski et al. 1992), require a pH manipulation, and are selective for hydrophobic constituents. Freeze-drying is excellent for collecting a small fraction of dissolved organic matter; however the process is time-consuming and also collects nonvolatile salts thereby necessitating an additional desalting step. Another method used to separate proteins from free amino acids and small peptides is trichloroacetic acid precipitation (Smucker and Dawson 1986; Nguyen and Harvey 1994; Mayer, Schick et al. 1995) however this step generates low yields.

Solid phase extraction using silica capped with octadecyl (C18) columns is often used for desalting and concentrating organic molecules. The literature has shown that C-18 columns are more efficient than XAD resins (Amador, Milne et al. 1990), and have been used successfully to extract herbicides (Ferrer, Barcelo et al. 1999), metal-organic complexes (Mills, Hanson et al. 1982), biodegradation products (Bielicka and Voelkel 2001), dissolved lignin (Louchouarn, Opsahl et al. 2000), and dissolved organic matter (Amador, Milne et al. 1990) from natural waters. Kim, Simpson et al. (2003) found that C-18 disks can retain approximately 60% of dissolved organic matter and, using electrospray ionization- mass spectrometry, confirmed that this concentrate retained the distribution of functional groups in the original sample although these results are dependent on what compounds passed through the filter. In the present research, components of dissolved organic matter, such as humics, may interfere with further

protein analysis. C-18 columns were tested to examine how useful they are in the extraction and concentration of proteins from natural waters for the present research.

Ultrafiltration can be used to fractionate DOM according to molecular size rather than chemical properties (Benner, Pakulski et al. 1992). The smallest molecular weight cutoff is usually 1000 Da and is therefore more appropriate for the concentration of dissolved proteins, while excluding small peptides and free amino acids. Ultrafiltration using 15 mL centrifugal filters was tested for efficacy in extracting and concentrating proteins in natural waters.

2.1.2 Quantification

Colorimetric assays

There have been a number of techniques employed to measure dissolved protein concentrations. Every quantification method is limited in the sense that the protein used for the calibration of the method cannot mimic the complexity of heterogeneous proteins in natural waters. The Lowry assay (Lowry, Rosebrough et al. 1951) is historically the most widely cited method for protein determination in a biological medium. This method is sensitive to many interfering compounds, including free amino acids (Peterson 1979), requiring extraction and filtration steps to separate free amino acids and proteins. Nonionic detergents and buffer salts can interfere by forming insoluble precipitates with Folin-Ciocalteau reagent. The assay reagents are unstable in alkaline conditions, and therefore exact timing is essential to this measurement. Other assays, such as Coomassie Blue assay (Setchell 1981), requires the separation of low molecular weight materials

from protein, because the assay measures small peptides as well as proteins (Mayer, Schick et al. 1986). Nguyen and Harvey (1994) successfully quantified particulate protein in aquatic systems using the more recent bicinchoninic acid (BCA) assay, another colorimetric assay which uses copper complexes to bind to peptide bonds, but it is uncertain how accurate the method is for dissolved proteins in the presence of compounds in natural waters. The BCA assay involves the formation of a Cu^{2+} and peptide linkage complex (Smith, Krohn et al. 1985). The reduced Cu^{1+} then reacts with the BCA assay reagents, bicinchoninic acid. The product of this reaction is a chromophore that can be measured by spectrophotometric analysis. The assay will vary between proteins based on the exposure of protein peptide bonds to the copper solution and availability of easily oxidizable amino acids, such as tyrosine and tryptophan.

The BCA assay can be effective in measuring particulate proteins (Nguyen and Harvey 1994). Particulate proteins are easier to quantify than dissolved proteins because they can be removed from interferences in seawater by filtration and are present in higher concentrations in natural waters. The BCA assay has high reagent stability and is less susceptible to interferences from other compounds. The superiority in BCA measurements of particulate proteins in aquatic systems led to the testing of the assay for the measurement of dissolved protein concentrations in natural waters in this chapter.

Size exclusion chromatography (SEC)

Knowledge of the size distribution of proteins and peptides is important for understanding the steps in protein degradation processes. Size exclusion chromatography, also known as gel filtration chromatography, separates proteins by

nominal molecular mass. The column matrix contains pores that permit buffers and smaller proteins to enter, but excludes larger proteins and complexes, the size of which can be varied (Figure 2.1). Larger proteins are forced to migrate around column particles and elute first, while smaller molecules can penetrate the gel matrix during passage and therefore elute later.

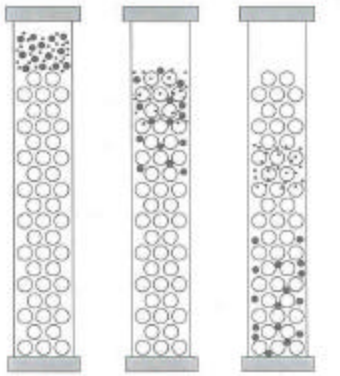


Figure 2.1 A diagram illustrating the principle of size exclusion chromatography. Open circles represent the column matrix, closed circles represent proteins of various molecular sizes. The three figures show the separation of large molecules from small molecules over time. As shown, the large molecules quickly travel between the beads of the column matrix, while the small molecules become trapped within the pores of the beads. Figure take from Bollag, Rozycki et al. (1996)

The elution of proteins is typically monitored using fluorescence (Figure 2.2). The intensity of the eluting peaks is integrated for protein quantification. Size-exclusion chromatography as a method of quantification is appropriate for the present research because it can be used as a measure for specific protein fraction quantities as well as to separate protein size fractions for further analysis. Other advantages are high protein resolution, minimal analysis time, minimal sample loss, and highly reproducible results (Bollag, Rozycki et al. 1996).

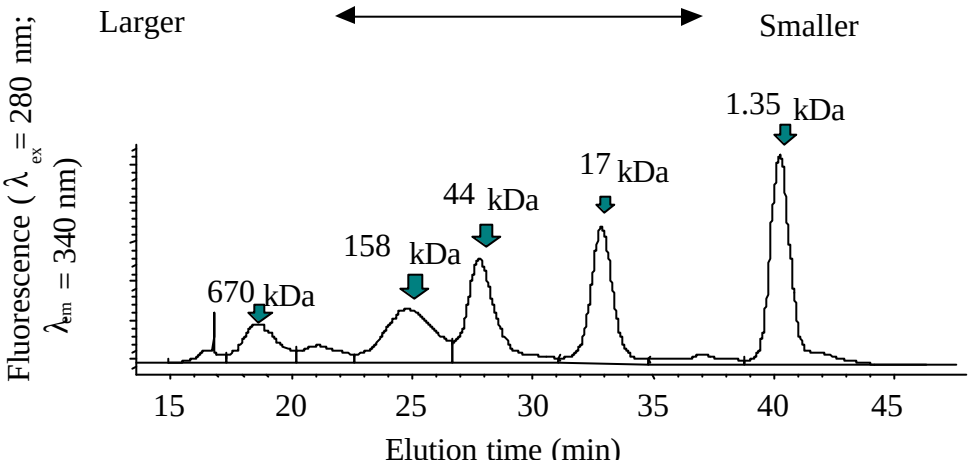


Figure 2.2 Chromatogram demonstrating elution order of proteins in size-exclusion chromatography

An important caveat for size-exclusion column separations is that it assumes all proteins are globular in shape and therefore elute at the same flow rate (Bollag, Rozycki et al. 1996). To eliminate protein shape as a variable, chaottropic agents such as guanidine are added to the sample prior to size-exclusion chromatography (Bollag, Rozycki et al. 1996). The tertiary structure is reduced, in other words, unfolding of the protein occurs to give a more reproducible molecular weight. A high concentration of guanidine·HCl can become problematic when working with proteins with numerous subunits, such as ribulose-1,5-bisphosphate carboxylase (Rubisco), because it can denature them to the point of dissociation of the protein into its subunits (Jiang, Wang et al. 1997).

A second potential disadvantage of SEC is that it separates proteins by molecular mass, not by size. The results are partly influenced by compound interaction with column material, such as charge repulsion, hydrogen bonding, and hydrophobic interactions. Although the addition of guanidine·HCl is necessary for protein shape consistency, it denatures or unfolds the proteins (Creighton 1993), exposing the interior amino acids to the column material. In this way, a protein may be eluted differently due to increased interactions with column material. Size-exclusion chromatography was tested for efficiency in quantification and separation of dissolved proteins in aquatic systems.

2.1.3 Use of Surfactants

Detergents are amphiphilic molecules with high solubility in water. They form clusters of detergent molecules, micelles, with hydrophilic heads pointing towards the aqueous solution (Bollag, Rozycki et al. 1996). Detergents also protect hydrophobic portions of proteins from coming into contact with other proteins and forming aggregates. In order to form micelles, the critical micelle concentration must be reached, which is defined as the lowest concentration of detergent required to form micelles in solution (Bollag, Rozycki et al. 1996). Because simple dilution will dissolve the micelles, detergents can easily be removed through filtration prior to further analysis.

Protein aggregation can be an important issue in natural environments, so two detergents were tested for surfactant efficacy, sodium deoxycholate and sodium dodecyl sulfate. The critical micelle concentration for sodium deoxycholate is 0.57% (Bollag, Rozycki et al. 1996). Some potential issues with the use of deoxycholate are its precipitation at low pH and in the presence of divalent cations (Bollag et al. 1996). Sodium dodecyl sulfate with a critical micelle concentration of 0.03% is a widely used detergent not known to be affected by divalent cations; however, sodium dodecyl sulfate is known to be very sensitive to salt concentrations and a shift to lower critical micelle concentration is observed at higher salt concentrations (Bollag, Rozycki et al. 1996). The efficiency of detergents in the removal of dissolved protein from surfaces involved in processing and preventing adsorption of proteins was examined, as well as the effect of detergent on the BCA assay for quantification of proteins.

2.2 BCA assay for dissolved protein quantification

2.2.1 *Description of BCA Assay Protocol*

A protein standard calibration curve was made using 100 µL of various concentrations of BSA (purchased commercially from Sigma without further purification) in triplicate. Each concentration is made by sequential dilutions of a stock solution of BSA (2 mg mL^{-1}) in 0.16% deoxycholate made in distilled, deionized water. Total protein was measured by the BCA assay (Smith, Krohn et al. 1985). BSA concentrations for the formation of a standard curve are as follows: 0 ng,

100 ng, 200 ng, 400 ng, 800 ng, and 1.6 μg. The unknown samples in 100 μL aliquots are measured in triplicate to correct for assay and sampling variance.

A 50:1 mixture of Reagent A (Bicinchoninic acid solution): Reagent B (copper sulfate solution) was prepared with 2 mL is added to each tube containing 100 μL of the sample being measured. The samples are then covered and placed in a 60°C incubator for one hour following the protocol developed for particulate protein measurements (Nguyen and Harvey 1994). Absorbance in each sample is read at 562nm with a Shimadzu UV-120-20 spectrophotometer.

2.2.2 Potential Interferences to the BCA Assay

A natural estuarine water incubation with an addition of BSA was run to examine interferences on absorbance readings of the BCA assay. Water was collected from the lower Delaware Bay and filtered through a GF/F (0.7 μm) filter. A BSA addition of 1 mg L^{-1} was added to the experimental treatment. This experiment was run over a 48 hour time period and aliquots were collected at 0, 24, and 48 hours in duplicate. A distilled water blank was run as a control. Each aliquot was ultrafiltered using a 1 kDa membrane. Changes in total protein concentrations were followed in the >1 kDa and < 1 kDa fraction using the BCA assay

Surfactant Testing

The effects of deoxycholate and sodium dodecyl sulfate on the BCA assay were tested using 0.2 µm filtered water from the lower Delaware Bay. With a duplicate of each treatment, deoxycholate or sodium dodecyl sulfate detergent was added to dilutions of natural water with deionized, distilled water as shown in Table 2.1. From each treatment, 1 mL was removed and sonicated for 5 minutes, then refrigerated overnight. The BCA assay was performed as described and absorbance recorded.

Detergent	Detergent Concentration (%)	Nanopure: 0.2um filtered estuarine water	Absorbance (nm) λ= 562 nm
None	0	Nanopure	0.223
None	0	Natural water	0.205
DOC	.08	1:1	0.19
DOC	.15	1:1	0.209
SDS	0.007	1:1	0.204
SDS	0.15	1:1	0.207

Table 2.1: Detergent, 0.2um filtered estuarine water, and nanopure water concentrations used to examine the interference effects of detergent and natural water on the BCA assay protein measurements.

Effects of salinity on the BCA assay

The effect of salinity on the absorbance readings of the BCA assay was tested. Duplicate BSA standard curves were carried out using natural water diluted with deionized, distilled water for a range of salinities with deoxycholate present (0, 2, 4, 6, and 8 ppt) and without deoxycholate (0, 4, 8, 11, 15 ppt). The effects of salinity on

the detergent, deoxycholate, and precipitate formation were also examined by running the range of salinities twice, once with detergent (final concentration 0.16% deoxycholate) and again in the absence of detergent. A 1 mL aliquot was removed from each salinity for analysis. To the samples with deoxycholate, 1 mL of 0.3% deoxycholate was added for a final concentration of 0.16% deoxycholate, diluting the salinity of these samples in half as indicated. Each water sample was sonicated for 5 minutes and refrigerated overnight. Standard absorbance curves were made using BSA stock solution (2 mg mL^{-1}) diluted with each water treatment. The BCA assay was run as described in the protocol and absorbances recorded.

2.2.3 Use of the BCA assay be used for quantification of dissolved proteins in natural waters

The BCA assay is known to be affected by free sugars (Smith, Krohn et al. 1985) and humic substances (Nguyen and Harvey 1994), both of which are present in aquatic systems. Nguyen and Harvey (1994) successfully used the BCA assay to quantify particulate proteins in natural waters. In order to examine if the BCA assay can also be used for dissolved protein quantification in natural waters, the effects of interferences on absorbance readings were investigated.

In the experiment to investigate potential interferences of the BCA assay, a negative absorbance was observed when comparing the natural water (1:1 diluted with deonized, distilled water) absorbance (0.206) to the deionized, distilled water absorbance (0.224). It is also important to note that in the presence of deoxycholate,

there is an even greater shift to lower absorbance (0.190). However, results from an extraction process (Section 2.4.3) show that deoxycholate increases the yield of BSA from concentration processes by 40%. Since deoxycholate is necessary, the standard curve should be run in the natural water used for the incubation and with the same concentration of deoxycholate that will be present in the samples.

When the incubation aliquots are examined after ultrafiltration it was apparent that these changes do not account for the total change in absorbance (Figure 2.3). A majority of the interference occurs from compounds larger than 1 kDa. While small peptides will still be present in this fraction, known interferences such as free amino acids would also exist in the smaller fraction. Humics are one candidate for the larger

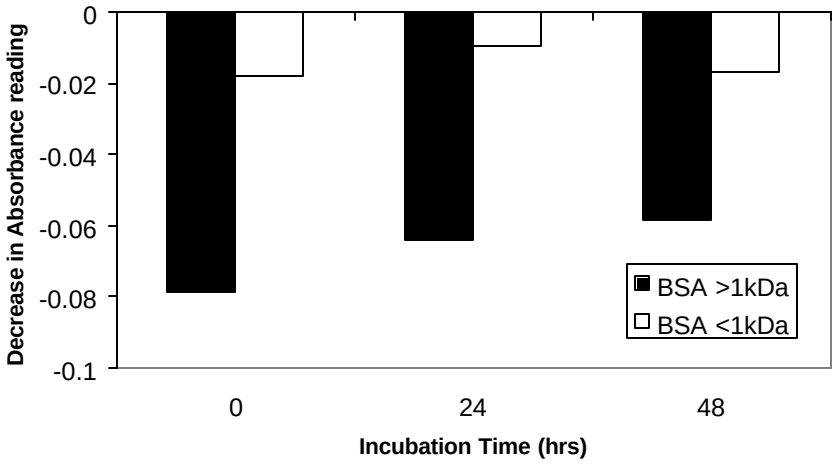


Figure 2.3 Time course of BCA assay 562nm absorbance change from blank (1:1 natural water: 0.16% DOC) measured using the BCA assay after ultrafiltration over time.

fraction, and could account for some negative absorbance. It is possible that ultrafiltration could form complex aggregates of organic matter, trapping detergent molecules and humics, which interfere with the BCA assay. Simple quantification of dissolved proteins using the BCA assay combined with the concentration process necessary to detect dissolved proteins does not appear a feasible option. However, an important result of this experiment is the demonstrated need for a concentration and filtration step prior to further analysis to remove small interfering substances that could affect other analyses used for characterization.

Choice of Surfactant

Due to protein aggregation in natural waters and potential adsorption of proteins to various substrates, the addition of a surfactant is necessary. Each surfactant examined had different characteristics as described above. The two detergents chosen, deoxycholate and sodium dodecyl sulfate, are known not to interfere with the BCA assay. A comparison of standard curves run in the two detergents show that both detergents have the same slope (Figure 2.4), but both lower the absorbance in the BCA assay analysis (0.014% sodium dodecyl sulfate blank intensity = 206 Abs., 0.16% deoxycholate blank intensity = 204 Abs., nanopure water blank = 223 Abs.). Because both detergents decrease absorbance, and the sensitivity of sodium dodecyl sulfate to higher salt concentrations, deoxycholate was chosen as the surfactant.

Salinity effects on the BCA assay

Smith, Krohn et al. (1985) found that NaCl does not affect the BCA assay; however the effect of other ions found in natural waters was not examined. As mentioned previously, divalent cations, which are plentiful in marine environments, are known to cause precipitation of deoxycholate (Bollag et al. 1996), and may be problematic when running the BCA assay. At salinity of 4 ppt and higher, a

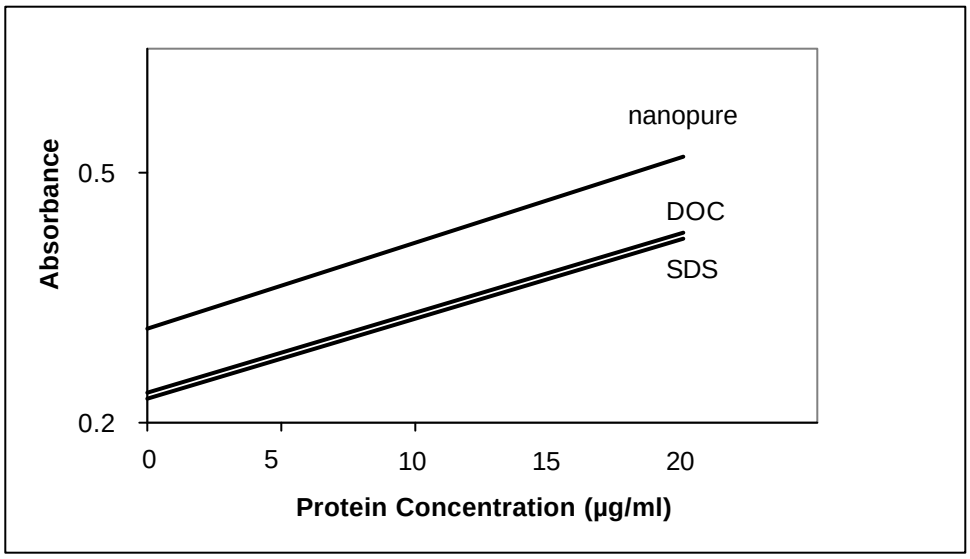


Figure 2.4 BSA standard curves run in distilled, deionized water (nanopure), in 016% DOC, and in 0.014% SDS for absorbance comparisons.

precipitate was in fact observed in the samples containing deoxycholate. This precipitate caused the BCA assay to be very variable. Therefore, to avoid this precipitate for accurate quantification, natural water would need to be diluted to lower the salinity below 4 ppt.

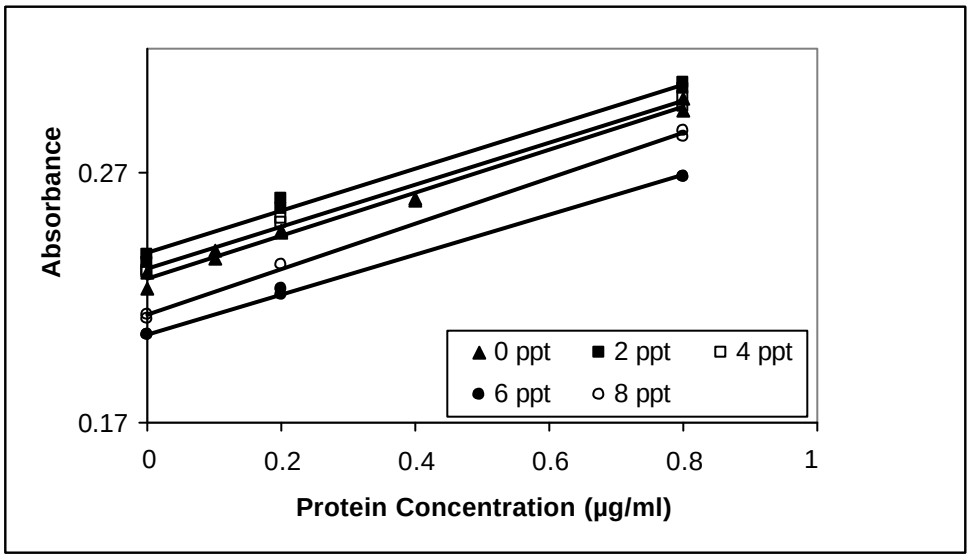
The effect of salinity on the BCA assay is shown in Figure 2.5. Although the regression is linear, there is a considerable decrease in absorbance as salinity increases. Results showed a 9% and 5% decrease in absorbance in the presence of deoxycholate at salinities of 6 ppt and 8 ppt, respectively. There was also a considerable decrease in absorbance in the absence of deoxycholate for salinities of 8 ppt and 15 ppt (6% and 20% for 8 ppt and 15 ppt, respectively). It is possible that the increase seen in absorbance in samples with deoxycholate could be due to the prevention of aggregation allowing BSA to open up exposing more sites to copper for binding.

At a salinity of 4 ppt and lower, there was no shift. This result indicates that the BCA assay can be effective if samples are diluted below 4 ppt. It is important to note that this absorbance could be an effect of salinity, but because natural water was used, the natural water itself may also be interfering with the BCA assay. Unfortunately, because dissolved proteins are already so low in concentration, and dilution would alter the water chemistry, it is unfeasible to use the BCA assay for dissolved protein in natural waters in the present research.

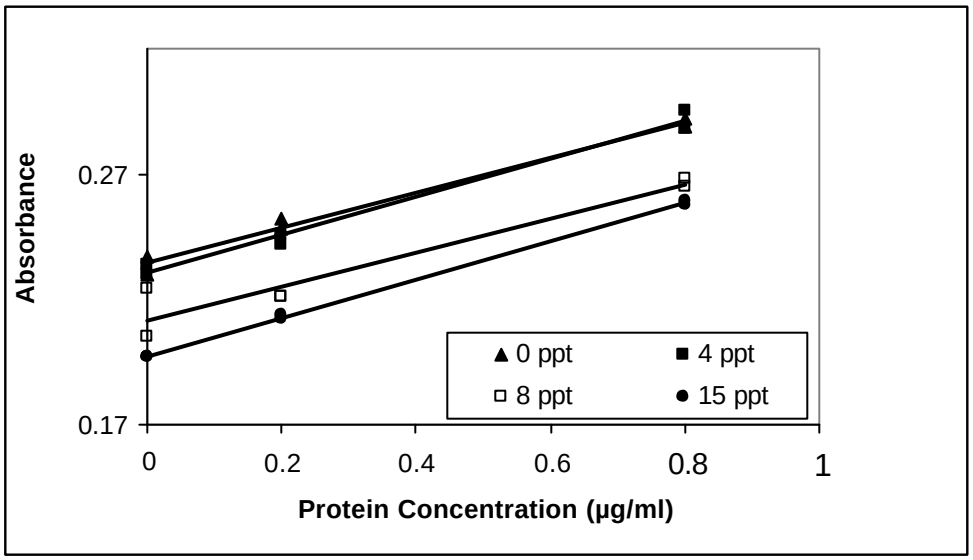
2.3 Size-Exclusion Chromatography for quantification and separation

2.3.1 SEC Protocol used

To separate and isolate individual proteins, a Superdex 200 HR 10/30 gel filtration column was used with a 50mM ammonium bicarbonate solution at a flow



(a)



(b)

Figure 2.5 Interference tests of salinity and detergent on Protein standard curves for bovine serum albumin (BSA) using the Bicinchoninic acid assay. Treatments included (a) Salinity and DOC effects on the BSA standard curve. Y-intercepts are as follows: 0 ppt: 0.2057; 2 ppt: 0.2379; 4 ppt: 0.2321; 6 ppt: 0.2057; 8 ppt: 0.2137; (b) salinity effects on the BSA standard curve. Y-intercepts were as follows: 0 ppt: 0.2278; 4 ppt: 0.2307; 8 ppt: 0.205; 15 ppt: 0.1973. Assay was performed in triplicate at three concentrations of BSA.

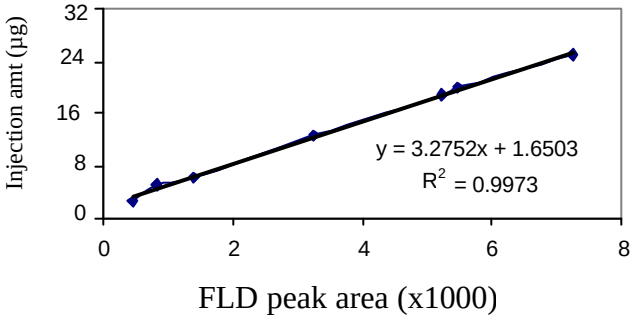
rate of 0.5 mL min^{-1} and injection volume of 100 µl. Protein concentration and elution is followed by fluorescence at λ_{ex} = 280 nm and λ_{em} = 320 nm. This method combines quantification and separation of modified products for further analysis into one step.

The BSA is quantified by integrating the BSA peak. To quantify unknown concentrations, first known quantities of BSA (2.5 µg, 5 µg, 6.25 µg, 12.5 µg, 18.75 µg, 20 µg, and 25 µg) are injected in sequential dilutions at equal volumes to develop a calibration curve (Figure 2.6a). A linear regression equation is used to interpolate the concentration from peak area. Because of the potential for small amounts of modified products at low concentration, (lower than 2.5 µg,) a separate calibration curve (0.5 µg, 1 µg, 1.75 µg, 2.5 µg) was also determined.(figure 2.6b)

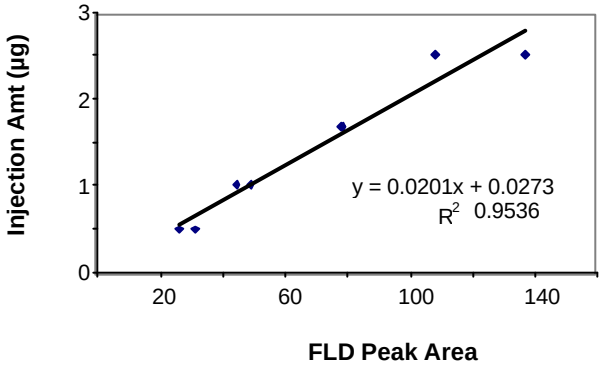
To estimate the apparent molecular mass of the proteins and protein fragments eluded, the column was calibrated before use with the following molecular weight standards: (Sigma) including aprotinin (M_r 65,000), cytochrome c (12,400), carbonic anhydrase (29,000), bovine serum albumin (67,000), and blue dextran (2,000,000) and (Bio-Rad) including thyroglobulin (M_r 670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1350). A regression was calculated based on time and these molecular weight markers (Figure 2.7). The regression equation was then used to estimate molecular weights of unknown proteins.

Potential interferences existing in natural waters were tested. Four treatments in duplicate were used and included (a) BSA, 0.16% deoxycholate, 0.15 M guanidine

and 0.7 µm filtered estuarine water, (b) BSA, 0.15 M guanidine, and 0.7 µm filtered estuarine water, (c) BSA, 0.16% deoxycholate, and 0.7 µm filtered estuarine water,



(a)



(b)

Figure 2.6 Examples of two protein standard curves for the model protein, BSA, using size exclusion chromatography- HPLC with fluorescence detection. (a) A standard curve with BSA concentration range of 2.5-25 µg; (b) a standard curve with BSA concentration range of 0.5-2.5 µg.

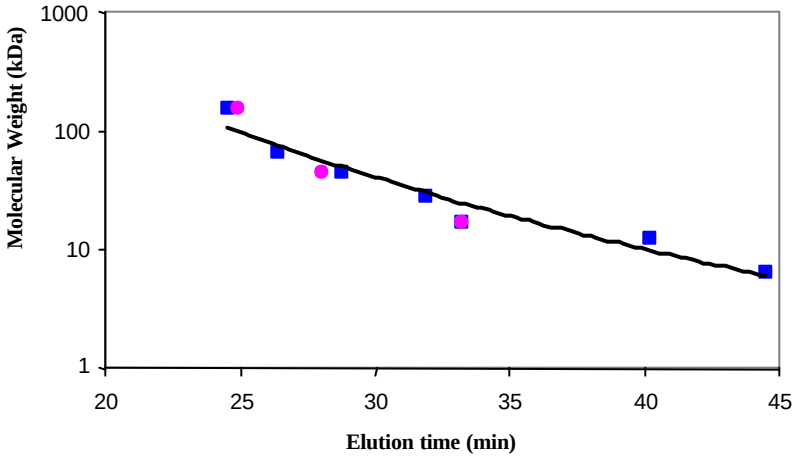


Figure 2.7. Elution time (Duplicate samples- square and circle) of various molecular weight markers used as a calibration of dissolved proteinaceous material. The void volume is indicated above respective retention time.

(d) 0.7 µm filtered estuarine water and 0.16% deoxycholate. Each mixture (15 mL) was sonicated, then left to sit for 1 hour to allow deoxycholate-protein micelle formation. Each mixture was then concentrated with an ultrafiltration centrifuge filter (15,000 x g , 20°C) to a volume of 150 µL. Both retentate and filtrate for each treatment was injected and separated by SEC to see if any of these compounds interfered with the fluorescence detection.

2.3.2 Results of SEC quantification

No compounds (deoxycholate, guanidine, or any components of estuarine water) were detected using HPLC-SEC fluorescence. Without deoxycholate present, however, the protein peaks produced by fluorescence were noticeably broader. The void volume (>1,000 kDa) was larger without guanidine·HCl addition to the sample

prior to HPLC injection, indicated aggregation of proteins. Guanidine·HCl is highly corrosive and rapidly deteriorates the condition of the HPLC and therefore cannot be added to the mobile phase. However, guanidine·HCl within the sample does not appear to have the same effect and assists in prevention of aggregation. Also, as described in Chapter 1, the presence of guanidine·HCl minimizes the concerns of different protein shapes effecting elution time. This quantification method proved to be superior to the present colorimetric assays by detecting low protein concentrations (= 1 μg mL^{-1}), producing consistent results, specifically quantifying BSA instead of total protein concentrations as in colorimetric assays, being unaffected by interfering compounds in natural waters, and allowing for the separation of protein products for further analysis.

Although SEC proved to be a superior method for quantifying dissolved proteins in estuarine and marine waters over the BCA assay, this method is not without disadvantages. A major limitation of SEC is the variability in peak intensity between different proteins. Although when modified products are seen, they can be isolated for further characterization, the modification may have changed its reactivity to the fluorescence detector and this absolute quantification is not guaranteed. A second issue is the selectivity of protein molecular weight observed. Because dissolved protein modification product sizes are unknown, the SEC column used in the present research can separate a range of protein sizes from 1 kDa to 1000 kDa. This broad range decreases the sensitivity of the column to specifically determine protein MW. In order to look in greater detail at specific molecular weight ranges, it

is necessary to compromise the breadth of detail seen in the examination of dissolved protein modification and degradation in natural waters.

2.4 C-18 columns for Extraction and Concentration

2.4.1 C-18 columns testing

C-18 columns are potentially useful in these studies because the model protein, BSA, is a hydrophobic protein, and therefore should be easily extracted from the column to quantify and further separate for modification analysis. Protein binding using C-18 columns (Varian Bond Elut LRC, C-18, 500mg) and the abilities of different solvents to elute dissolved proteins were tested. First, estuarine water was collected and filtered through a GF/F filter (0.7um) to eliminate particles. One mg L^{-1} of BSA was added to the water and allowed to stand for 1 hour to allow full dissolution of BSA. The C-18 columns were activated with 20 mL of methanol, and then 100 mL of sample was passed through each of three columns. A control sample of 30 mL containing no BSA was also passed through three separate columns for comparison of recoveries and elution contamination. In order to remove all remaining salts, 10 mL of nanopure water was passed through each column prior to elution. Each pair of columns (BSA and Control as a pair) was eluted with a different solvent system to test elution capabilities. The three solvent systems tested were 30 mL of acetonitrile, 20 mL of methanol, and 20 mL of 35% isopropanol, 35% acetonitrile, and 30% water in 0.5% glacial acetic acid (Reno Nguyen, personal communication). The eluent was collected and solvents were removed. The volume

within each flask was brought up to 10 mL with 0.16% deoxycholate to prevent loss of protein to adsorption on container walls. The BCA assay was run as described in assay protocol.

2.4.3 Results of C-18 Testing

An examination of different eluent systems showed that the isopropanol: acetonitrile: water eluent worked the best, giving a 70 ± 2% yield. Less protein was recovered from the columns using acetonitrile and methanol, yielding 30% and 7% for acetonitrile and methanol, respectively. Multiple replicates (not discussed here) have shown that the addition of the detergent, deoxycholate, increases the C-18 column recovery of total protein by 40%. With low concentrations of dissolved protein in natural waters, it is imperative to recover the most protein possible.

In all samples analyzed by the BCA assay after the concentration of protein using C-18 cartridges, there remained a negative absorbance (compared to deionized, distilled water) at 562 nm. These columns did not show the ability to remove small interferences, likely because they rely on hydrophobicity for sample retention. For the same reason, it is possible that if proteins are modified during an experiment, changes in protein hydrophobicity may occur, altering their affinity for the column. It is also seen from previous experiments that recoveries are low and variable, especially when dealing with low concentrations (<1ug ml^{-1}) of dissolved proteins in natural waters.

2.5 Centrifugal Ultrafiltration for extraction and concentration

2.5.1 Centrifugal Ultrafiltration testing

The efficiency of the ultrafiltration centrifugal filters (Millipore Ultrafree, 5K NMWL membrane) was tested for removal of salts, retention of proteins, and ease at which proteins could be removed from the membrane once concentrated. Both deionized, distilled water and 0.7 μm filtered estuarine water were spiked with BSA at a concentration of 2 mg L^{-1}. There were three different methods examined for removal from the centrifugal filter. The effect of detergent on recovery from the ultrafiltration centrifuge filter was also tested in this experiment. Although previous results have shown deoxycholate to aid protein recovery from glass (Section 2.4.3), it is unknown if protein recovery from centrifugal filters is also affected. To examine the effect of detergent, each method was run with and without deoxycholate present in the sample as it was being concentrated (Figure 2.8).

2. 5.2 Results of Ultrafiltration testing

Results from Ultrafree testing (set-up Figure 2.8) show that after sample concentration, a 10 mL nanopure rinse, and then followed by 5mL of 0.16% deoxycholate recovers the most BSA from the filters (98% yield) compared to without deoxycholate (93% yield) (Table 2.2). Again deoxycholate is proven to be necessary for higher recoveries of protein. Deoxycholate was not needed as an aid in concentration, but was necessary after concentration to remove the protein from the

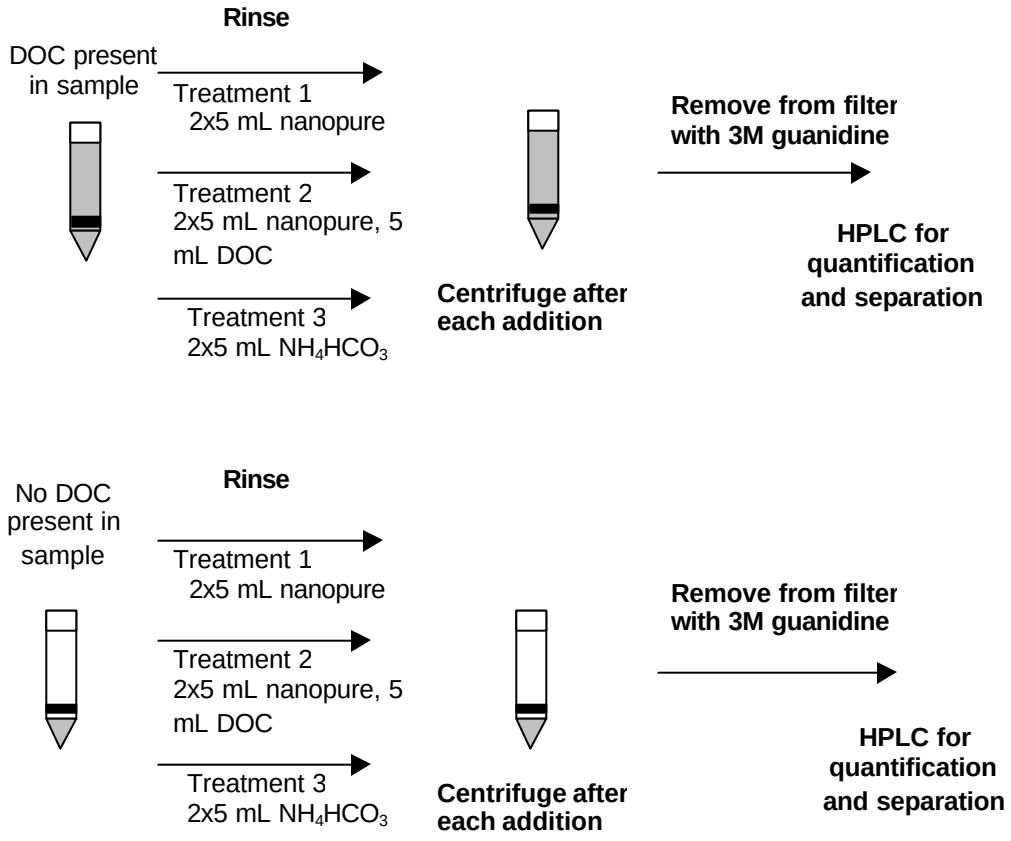


Figure 2.8 Experimental set –up to examine the method giving the highest yield for concentration by ultrafiltration centrifugal filters, followed by the removal of protein from the filter after concentration. The above experiment was run in duplicate with BSA added to deionized, distilled water (nanopure), and then BSA added to river water.

filter. When deoxycholate is added to the sample, the deoxycholate forms a precipitate that causes a decrease in BSA recoveries as observed in previous experiments. In this case, a simple rinse of the filter to dilute seawater removed precipitates that formed worked well, making this method more useful than C-18 columns in which the interferences could not be removed. There was no difference in the recovery of in deionized, distilled water (96%) and in natural Patuxent River

water (98%) (Table 2.2). This result suggests that interactions of proteins with dissolved organic matter and other substrates found in natural waters do not decrease the recovery of BSA from the centrifugal filters. The best recoveries for filtered natural water were those samples that contained no deoxycholate until after the sample was rinsed with nanopure to remove interfering compounds. However, transferring the concentrate to a vial for further analysis using deoxycholate prevented aggregation of proteins and loss by adsorption to the centrifugal filter membrane, thereby increasing yields.

Ultrafiltration concentrates material based on size instead of chemical properties, as in C-18 columns. This method for extraction removes small interfering compounds while retaining any compound above the size of the membrane pores. Centrifugal ultrafiltration also does not require pH changes throughout extraction as in C-18 columns, which is an advantage, because adjustments in pH could change

	no DOC		with Detergent	
	ug/ml	yield	ug/ml	yield
Actual BSA conc.	8.00		8.00	
BSA in nanopure	7.45	93.09	7.73	96.3
BSA in river water	7.48	93.51	7.80	97.58

Table 2.2 Testing Ultrafree centrifugal filters efficiency in different water types. Recovery of BSA added to deionized, distilled water (nanopure) and in Patuxent River water in the presence and absence of deoxycholate (DOC).

chemical associations and structures of proteins within the sample, therefore adding another unnatural variable. Ultrafiltration also allows control over the molecular weight range of proteinaceous material analyzed and this better defines a dissolved

phase by the size of the membrane. For these reasons, centrifugal ultrafiltration was chosen as the superior method for extraction and concentration of dissolved proteins in natural waters.

2.6 Conclusions

Dissolved proteins in natural waters are difficult to quantify due to their low concentrations, the complexity and heterogeneity of naturally occurring proteins, and the presence of humics, minerals, and other compounds in seawater that interfere with protein measurement. In the present study, a model protein, BSA, was added to natural waters and examined in order to avoid the complexity of naturally occurring proteins. Even with the use of a model protein, there were many issues in the extraction, concentration, and quantification of BSA. Two extraction and concentrations were tested and two quantification methods were tested for use in investigating dissolved proteins in natural waters.

For extraction and concentration, the C-18 columns showed low and very variable protein recoveries (from 50-70%), even with the relatively hydrophobic protein BSA. The C-18 columns are nonspecific in the extraction of proteins from natural waters, because they extract all hydrophobic material. Since it is unknown what chemical and structural changes occur to the protein in these natural water incubations, the C-18 column extraction efficiency of modified protein products remains uncertain. Instead, centrifugal ultrafiltration filters were chosen for the extraction and quantification of dissolved proteins in natural waters. Large quantities

(>100 mL) could be concentrated to very small volumes (150 µL) and the recoveries were extremely high. The filters could be rinsed after concentration to remove interferences smaller than 5 kDa that may hinder with further analysis.

The BCA assay had been successfully used for the quantification of particulate proteins in natural waters; however this was not the case for the low concentrations of protein found in the dissolved phase. The small compounds that are assay interferences in natural seawater can be removed from the particulate phase easily by filtration. Due to the similarity in size of dissolved proteins and these interfering compounds found in natural waters, it is impossible to separate them entirely, making the BCA measurements highly variable. Also, the complex organic matrix in which proteins exist may trap the dissolved proteins and interferences, either blocking the protein from exposure to the assay or increasing the difficulty of separation of the proteins from interferences.

Size-exclusion chromatography proved effective in the quantification of dissolved proteins in natural waters. Unlike colorimetric assays which are only capable of quantifying total protein, it is a very specific method for the quantification of BSA. It therefore gives a deeper understanding of what modifications occur to the added protein within the natural water incubations in the present research. This method also allows for separation based on protein size, allowing for further analysis to gain additional understanding of the degradation process of BSA.

Chapter 3: Protein Modification and Degradation in Estuarine Environments

Section 3.1 Introduction

Proteins are important components of the dissolved nitrogen cycle. Most dissolved nitrogen in aquatic environments is believed to be in the amide form (Knicker and Hatcher 1997; McCarthy, Pratum et al. 1997). Because most nitrogen in plankton (believed to be the biggest source of DOM in marine waters) is present as protein (Lourenço, Barbarino et al. 1998), it can be hypothesized that much of the organic nitrogen in marine environments is proteinaceous material (further discussed in Chapter 1). Although proteins have been considered labile in marine environments and therefore not readily preserved (Hollibaugh and Azam 1983), evidence now suggests that preservation of proteinaceous material in aquatic environments does occur (Nguyen and Harvey 1997; Pantoja, Lee. et al. 1997; Nguyen and Harvey 1998). There remains little understanding of the proteins that may be preserved and the microbial processes responsible for utilization of dissolved protein.

The effects of different environments on amino acid composition in natural water have been extensively investigated (Keil and Kirchman 1991; Cowie and Hedges 1992; Hubberten, Lara et al. 1994), as well as the potential for dissolved proteins to appear in different waters (Tanoue 1992; Tanoue 1995; Tanoue, Ishii et al. 1996; Nguyen and Harvey 1998). Many models have been developed to describe uptake of proteins by bacteria. It is most commonly believed that extracellular

enzymes are released to hydrolyze proteins, and the products are either taken in by bacteria or remain in the environment (Mayer, Schick et al. 1995). It is currently being debated whether intermediates created during bacterial degradation of protein can be found in marine environments. Hollibaugh and Azam (1983) did not find intermediate peptides during the degradation of BSA; however, Keil and Kirchman (1991) and Pantoja and Lee (1999) did observe what they considered intermediates during protein degradation. Despite the increased knowledge of the potential for protein preservation, the effects of complexity and variation in aquatic systems on degradability of dissolved protein are not well understood.

There are many theories on general preservation pathways for proteins in aquatic systems as reviewed in Chapter 1. One theory is that some dissolved proteins like membrane proteins (Nagata, Fukuda et al. 1998) and porins (Tanoue 1995) are unavailable to bacteria and thus are selectively preserved from bacterial degradation. Abiotic processes, such as condensation reactions, have also been suggested as a preservation mechanism (Hedges 1978; Nagata and Kirchman 1997). Other recent evidence suggests that a mechanism of preservation may be sorption to mineral surfaces or other organic matter (Hedges and Hare 1987; Lee and Ruckenstein 1988; Kirchman, Henry et al. 1989; Keil, Montluçon et al. 1994). This process may occur through hydrophobic interactions which would stabilize proteinaceous material through aggregation (Nguyen and Harvey 2001). Another very likely, but less studied process of preservation is protection by encapsulation within a complex organic matrix (Knicker and Hatcher 1997).

The major focus of this chapter is the examination of the degradation and potential modification of protein during bacterial processing. We investigated the mechanism of dissolved protein degradation and modification by examining structural changes in a model protein added to natural water incubations. A time course experiment was performed to examine structural characteristics of intermediate peptide products formed from a known intact protein. Experimental incubations were used to provide a comparison of the protein degradation and/or its chemical modification by microbial communities in different aquatic environments to assess degradation variability. Dissolved protein added to incubations was subsequently collected and analyzed using size exclusion chromatography to follow chemical modifications based on molecular size. Amino acid analysis was used to determine if alterations in molecular weight included changes in amino acid distribution or random losses during hydrolysis. Our data demonstrate that although protein is utilized rapidly in the presence of natural microbial communities, there is a small fraction of dissolved protein that is modified through the degradation process, and these intermediates may play key roles in material cycling and preservation, and microbial ecology by further explaining the contribution of dissolved protein to the microbial loop and the larger aquatic food web.

Section 3.2 Materials and Methods

3.2.1 Protein Degradation Incubations

Patuxent River Incubations

Biotic and abiotic effects on protein degradation were studied in bottle incubations with natural waters. A treatment and two controls were used. These included an experimental incubation containing the natural bacterial community, an abiotic incubation sampled at specified time points, and an abiotic incubation which was not opened or sampled until the end of the incubation (unsampled contamination control) to avoid any contamination while sampling (See Figure 3.1 for visual). Incubation water was collected off of the Chesapeake Biological Laboratory pier

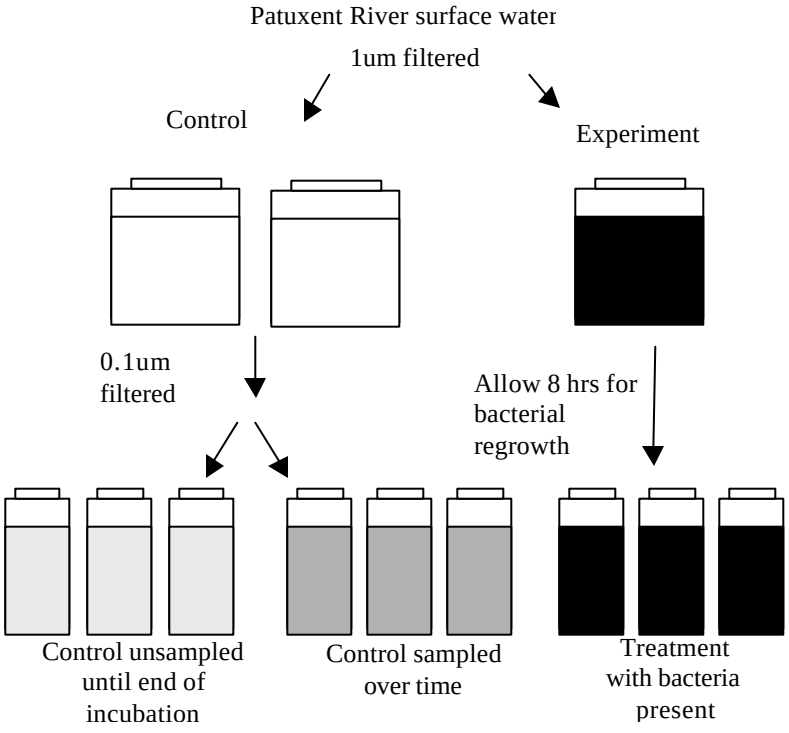


Figure 3.1 A schematic of the Patuxent River incubation protocol used to follow protein alteration.. Incubations were filtered through filters as shown.

from the Patuxent River, USA and separated into nine 1 L HDPE containers for three replicates of each incubation, then filtered through a 47mm (1 µm pore size, GF/B) filter. The bacterial community present in the experimental treatment was given approximately 8 hours to recover before addition of the model protein, bovine serum albumin (BSA). Each treatment was spiked with 5 mg L^{-1} BSA. Biotic incubations were sampled at specified time points. The abiotic controls were filtered through a 0.1 µm polycarbonate filter using autoclaved equipment and containers.

The influence of abiotic and biotic protein degradation was investigated by measuring BSA concentration change over 92 hours. Biotic and control incubations were sampled at 0, 12, 24, 36, 48 and 92 hours with bacterial counts taken at each time point (described in Section 3.2.2). From each incubation, 30 mL was removed and concentrated as described in the following method sections.

Field studies

Surface water samples were collected on the Delaware Bay, USA and Chesapeake Bay, USA, at 3 stations including a freshwater marsh site, a site in the high chlorophyll region near the mouth of the Delaware bay (lower Delaware Bay), and an anoxic site in the mesohaline portion of Chesapeake Bay, USA. At each site, two 20 L carboys were used to collect surface water and the water was filtered through a 0.2 µm polycarbonate filter to remove all particles including phytoplankton and bacteria. To the 0.2 µm filtered water a 10% innoculum of 3 µm filtered water, was added containing the natural bacterial community. Bovine serum albumin (1 mg

L^{-1}) was added to the carboy containing natural bacterial assemblage (see Figure 3.2). An aliquot of the 0.2 µm filtered surface water was used as a control. The experimental incubations were sampled at 12, 24, 36, and 48 hrs, with aliquots removed and frozen at –70°C until concentration and analysis. From each sample, 60 mL was removed and processed as described below.

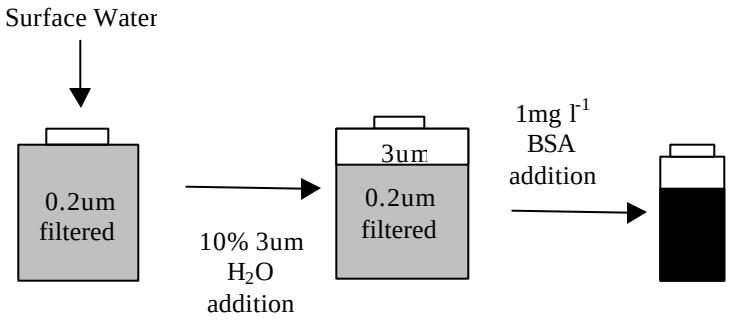


Figure 3.2 A schematic of protocol used for incubations (marsh, lower Delaware Bay, Chesapeake Bay) for the comparison of protein degradation between environments.

3.2.2 Bacterial Abundance in Patuxent River Incubations

In order to ensure there were no bacteria present in the sampled and unsampled abiotic incubations, and to determine that the bacterial population increased in the biotic incubation, bacterial abundance was measured at the beginning and end of the incubations, as well as specified time points during the incubation. Bacterial abundance was measured using DAPI counts on black polycarbonate filters (Porter and Feig 1980). From specified time points, 19 mL were removed and preserved by addition of 1 mL of 10% formalin in a neutral buffer (ca. 2% final

concentration). Vials were then refrigerated until filtration and counting.. For filtering, a black 0.2 µm polycarbonate filter was overlaid by a Millipore HA filter. For the abiotic and contamination control samples, 20 mL of incubation sample was filtered. For the experimental treatment, 1 mL of the sample was filtered. Ten fields were counted and an average for each sample was used.

3.2.3 Protein Concentration and Isolation

Dissolved protein amino acids were isolated and concentrated using ultrafiltration with centrifugal filters containing a nominal 5 kDa membrane (Millipore) as described in Chapter 2. Each aliquot was initially filtered through a 0.45 µm PDVP acrodisk membrane filter (Gelman Sciences) followed by concentration via the 5 kDa centrifugal filter. The samples were centrifuged (15,000 x g) at room temperature until concentrated to a volume of 150 µL. The filter was rinsed with 2 mL of 0.16% deoxycholate to minimize loss by adsorption to the centrifugal filter membrane followed by two rinses of 2 mL nanopure water to remove excess deoxycholate from the concentrate. The samples were transferred from the centrifugal filter using 350 µL of 3M guanidine·HCl in 50 mM ammonium bicarbonate buffer which was the buffer used for analysis. Additionally, the guanidine·HCl (3 M concentration) added to the concentrated sample was used to minimize possible aggregation of organic matter and, through denaturation, expose

more protein surface to acid hydrolysis in amino acid analysis described in Section 3.2.5.

3.2.4 Quantification and Separation of Proteins and Peptides

Size-exclusion chromatography was performed at room temperature under native conditions, i.e. the mobile phase does not contain a denaturing agent, using a Superdex 200 HR column (30 cm×10 mm, Amersham Pharmacia) operating with 50 mM NH_4HCO_3 mobile phase at 0.5 mL min^{-1}. Subsamples of 100 µL were injected and elution was monitored by fluorescence (λ_{ex} = 280 nm; λ_{em} = 340 nm). Peaks of interest were collected from the HPLC into 2 mL vials. Samples were stored at -70°C until prepared for further analysis. In size-exclusion chromatography, larger proteins are eluted first and size decreases with time (Figure 2.2). The performance of the column was calibrated using a set of known molecular weight standards from Sigma including aprotinin (M_r 65 kDa), cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (67 kDa), and blue dextran (2,000 kDa- void volume) and (Bio-Rad) including thyroglobulin (M_r 670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B_{12} (1.35 kDa). This calibration curve of these molecular weight standards (see Figure 2.7) was used to estimate molecular weights of unknowns based on retention time. To quantify modified proteins, known concentrations of BSA were injected in equal volumes to develop a calibration curve (Figure 3.3). This curve was then used to estimate concentrations of remaining BSA or amounts of modified proteins observed.

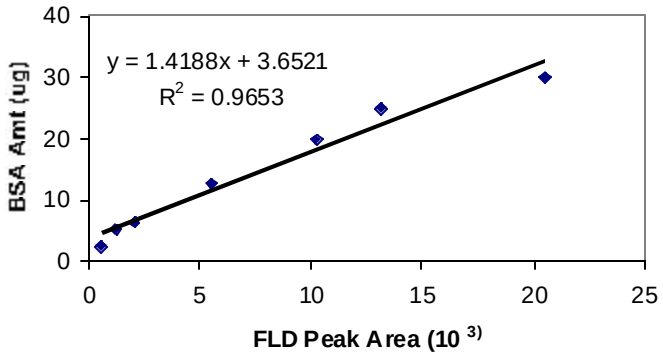


Figure 3.3 Calibration curve of known BSA concentration verses fluorescence area for determination of unknown protein concentrations.

3.2.5 Amino Acid Hydrolysis and Composition Analysis

Each concentrated sample was transferred to a 4 mL amber vial, a known amount (68.9 μM) of ?-methylleucine added as an internal standard, and then dried with nitrogen gas. The samples were then redissolved in 0.5 mL of sequanal grade HCl (Sigma Chemical Co), capped under nitrogen, and acid hydrolyzed at 150°C for 2 hours. Samples were dried under nitrogen at 45°C. The samples were subsequently derivatized to trifluoracyl isopropyl esters and analysed by gas chromatography and gas chromatography/mass spectrometry (GC/MS) as described in (Silfer, Engel et al. 1991). Separation of TFA isopropyl esters of amino acids was performed with a DB-5MS (J&W Scientific) fused-silica capillary column (60 m length, 0.32 mm i.d., 0.25 μm film thickness) using hydrogen (for GC) or helium (for GC/MS) as the carrier gas. The GC temperature was programmed from 50°C to 85°C at 10°C min^{-1}, then to 200°C at 3.5°C min^{-1}, and finally to 300°C at 10°C min^{-1} as method developed as (Nguyen and Harvey 1998) describes. Amino acid concentrations were determined

based on the internal standard and an external standard commercial mix of the amino acids (Sigma) used for determination of yields. Variable responses in individual amino acids were corrected for by comparison of areas of each amino acid to ?-methylleucine and calculation of recoveries. A BSA standard prepared in nanopure water was used to observe amino acid composition shifts from the original BSA added and also to test the variability of the hydrolysis method.

3.2.6 Ion Trap Mass Spectrometry Analysis

Mass determination measurements were performed with the Agilent Systems 1100 series ion trap mass spectrometer with an electrospray ionization interface. Protein samples (50 μg ml^{-1}) were infused at 400 μL min^{-1}, using a mobile phase of 50 mM NH_4HCO_3.with 0.1% formic acid. These MS conditions were used for all protein infusions are as follows: capillary voltage, 150-180 V; trap drive, 75-80 V; dry temperature, 350°C; nebulizer pressure, 15.0 psi. The mass spectra were registered in normal scan mode (m/z, 200-2000; scan time, 5 scans s^{-1}, maximum accumulation time, 50,000 μs, ICC target, 30,000). As the protein was being infused, the instrument was tuned for final optimization parameters to obtain the highest responses for each protein product examined.

Data was analyzed using Agilent software. Because proteins are multiply charged compounds, they acquire a range of charge states yielding a series of mass peaks for a single compound (illustrated in Figure 3.4). Each mass peak represents a different charge state. In this way, masses out of the m/z range of the instrument

(300-2200 m/z) can be measured. Deconvolution, provided by Agilent software, is then used to obtain a molecular weight from the many peaks produced (Figure 3.4). Deconvolution is a statistical method of transforming a multiply charged spectrum into a singly charged parent spectrum and calculating its molecular weight.

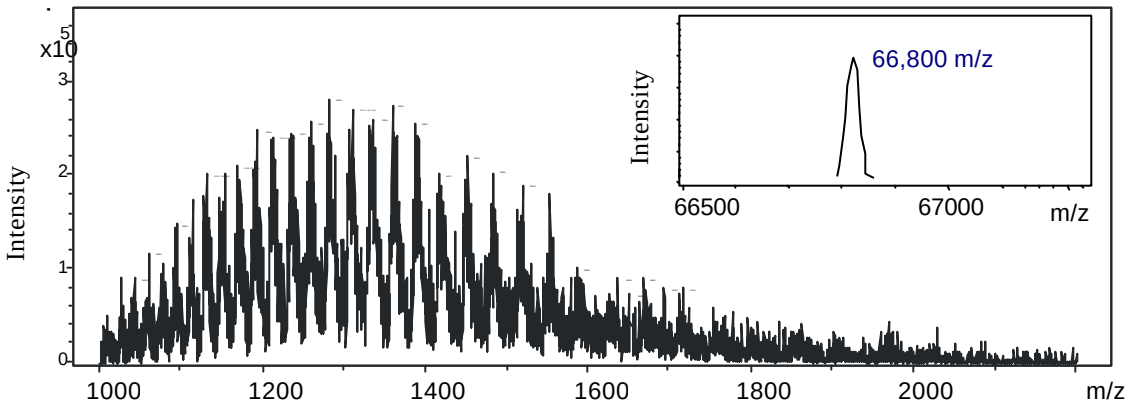


Figure 3.4. An example mass spectrum from electrospray ionization of a multiply charged protein introduced in its native state. The inset in the upper right corner is the peak after deconvolution for mass determination of the protein.

Section 3.3 Results

It is pertinent to first consider protein recovery following extraction, concentration and isolation processes. Of the total BSA added to the incubation, 20-70% was recovered, indicating that processing yields are highly variable. Recoveries of BSA from Ultrafree centrifugal filters alone were not as variable (93-96%, see Table 2.2), and the addition of BSA to either nanopure water or filtered Patuxent River, after concentration typically yielded over 90% (see Chapter 2). Although there was a high loss in protein during the incubations adequate proteinaceous material was

detected for additional analysis. As a consequence of these losses, qualitative findings must be emphasized.

3.3.1 Bacterial Degradation of Protein

The Patuxent River incubations compared protein degradation and modification in the presence of a natural bacterial assemblage (experimental treatment) and when bacteria were removed by filtration (abiotic treatment). Dissolved protein exhibited rapid degradation and modification in the presence of bacteria (Figure 3.5). The average BSA concentration of the three replicates at the beginning of the incubation was 2.05 μg mL^{-1}. At the end of the experiment, the average BSA concentration was 0.24 μg mL^{-1}. There was a rapid loss in BSA over the first 12 hours, with little change through the remainder of the incubation period.

The purpose of the sampled abiotic control incubations was to test the hypothesis that there would be very little dissolved protein modification and degradation in the absence of active bacteria. All the samples remained at approximately the same BSA concentration (0.5 ug mL^{-1}) until 48 hours (Figure 3.5). At sampled time point 92 hours, there was no BSA present in any of the replicates. An unsampled contamination control was run in parallel to be sure the sampling of

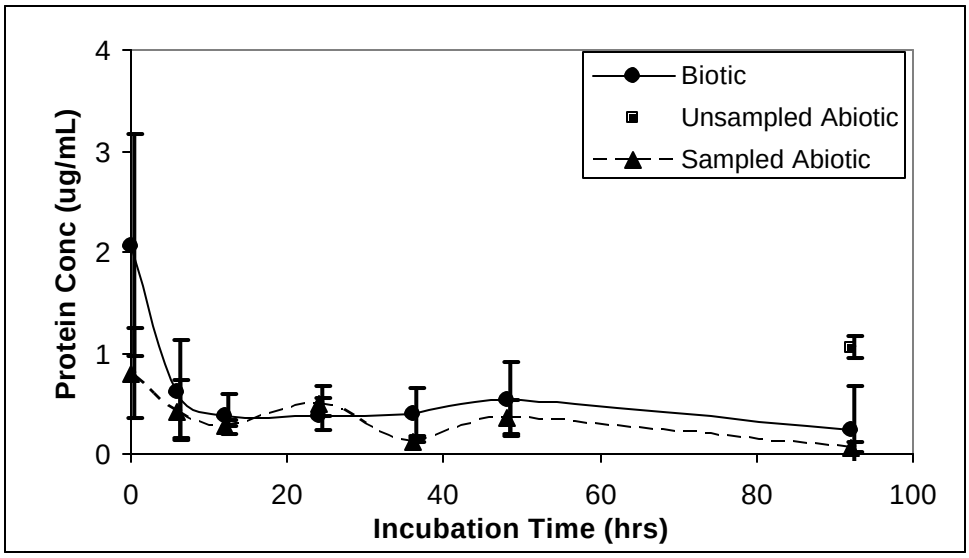


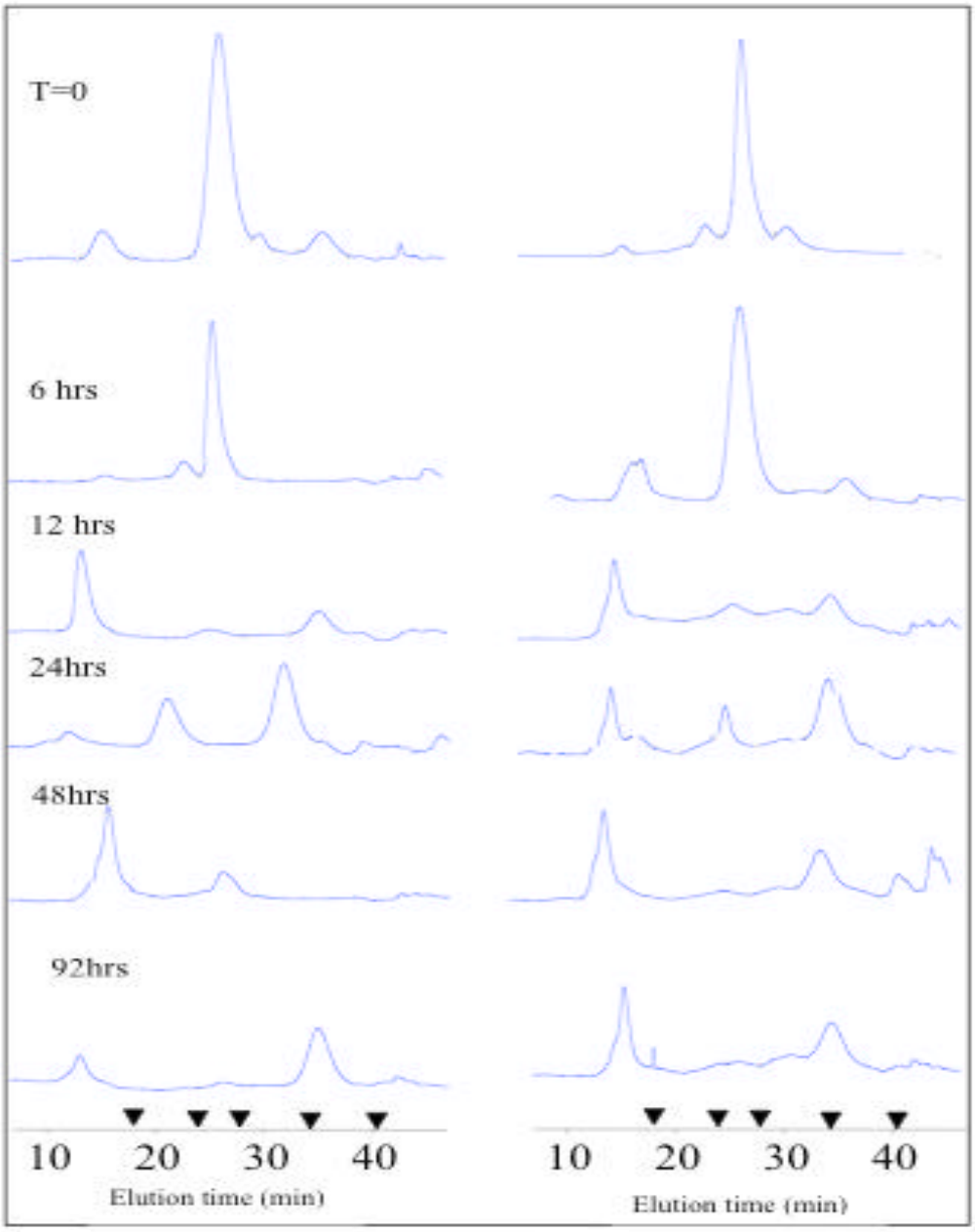
Figure 3.5 Time course of dissolved BSA concentrations in control (unsampled until 92 hours), abiotic control (sampled over time and contaminated), and in Patuxent River natural water incubation. Three replicates were averaged and error bars added.

the bottles did not affect protein measurements (Figure 3.5). The contaminated "abiotic" controls at 92 hours (average 1.5 μg mL^{-1}), indicates, along with contamination of control 2, that bacteria is the primary influence on protein concentration in natural water incubations.

Size exclusion chromatograms give a more detailed account of BSA modification and degradation and illustrate differences between the control (Figure 3.6a) and experimental (Figure 3.6b) incubations. A closer look at the size-exclusion chromatograms (Figure 3.6a) of the control incubations showed that instead of modified products being produced throughout the incubations, there is a disappearance of BSA entirely by 12 hours that can possibly be attributed to adsorption of BSA to the container walls. At 6 hours only BSA (67 kDa) and a peak

at approximately 120 kDa, which presumably would be the dimer of BSA, were observed. A larger void volume (= 1,000, kDa) peak appeared at 12 hours, giving an indication of aggregation of BSA in the incubations. At 24 hours there was a peak that would correspond to a molecular weight of 500 kDa. After 24 hours only the void volume remained in the incubation. The peak at 36 minutes found in almost all the chromatograms is almost certainly an artifact of the sample processing, because it was found in all chromatograms and further analysis did not show the presence of amino acids. In the unsampled abiotic control incubation, at 92 hours the BSA peak comprised of 88% of the detected proteinaceous material, with the rest existing as >1000 kDa molecular weight material throughout the entire incubation.

At the beginning of the experimental incubation only the BSA peak (67 kDa), BSA dimer (120 kDa), and a small intermediate peak at approximately 55 kDa were observed (Figure 3.6b). At 6 hours the BSA peak was observed to be broader, spanning a range of molecular weights of 35-120 kDa which is about 50 kDa wider than the BSA peak (˜52-100 kDa) observed at the beginning of the experiment. By 12 hours, the BSA peak had nearly disappeared, a sharp peak formed at >1000 kDa, and a number of small peaks of modified products were seen spanning the molecular weight range of 15-200 kDa. These low molecular weight products formed in the experimental incubation were not observed in the control incubations. At 24 hours a >1,000 kDa peak was seen in the experiment incubation and there was a shoulder that developed suggesting a slightly lower molecular weight than the void volume alone (approximately 600 kDa). The small lower molecular weight peaks were still observed at 24 hours. A small increase in BSA was observed, possibly indicating



a) b)

Figure 3.6: Time course of the a) control and b) experiment of the Patuxent River incubation. The time point is displayed on the chromatograms in the upper left corner. The chromatograms are resized (not shown to scale) to show peak locations and therefore do not indicate relative amount of protein present at each time point. Molecular weight standard elution times are indicated along the x-axis. The order of elution is as follows: 670 kDa, 158 kDa, 44 kDa, 17 kDa, and 1.35 kDa.

desorption of a small fraction of BSA from the container walls. After 24 hours the control and experimental chromatograms differed greatly. At 48 and 92 hours the experimental incubation showed very small amounts low molecular weight modified products remaining in the incubation. At both time points a predominant peak of >1000 kDa was observed, indicating that this peak is more refractory than the lower molecular weight products.

To further aggregate the data, the observed peaks were grouped into >BSA (100-1000 kDa), <BSA (2-52 kDa), and BSA (52-100 kDa) (Table 3.1). In the experimental incubation, the peaks with molecular weight (MW) higher than BSA increased over time from 0 hours (11.7%) to 24 hours (55.6%) then remained relatively constant throughout the remainder of the incubation. The BSA peak showed a constant decrease through the experiment from time 0 (76.5%) to 92 hours (8.6%). The low molecular weight products increased from 0 to 6 hours (from 11.8% to 51.3%), and then showed a sharp decrease until the end of the experiment to 8.6%.

	Experiment			Control		
Time (hrs)	% >BSA	%<BSA	% BSA	% >BSA	%<BSA	% BSA
0	11.7	11.8	76.5	9.7	5.8	84.5
6	37.0	51.3	11.7	52.9	0.0	47.1
12	41.0	30.6	28.4	38.1	18.9	43.0
24	55.6	21.4	23.0	22.3	0.0	77.7
36	61.5	19.5	19.0	51.5	0.0	48.5
48	65.2	20.7	14.2	45.4	0.0	54.6
92	56.2	35.1	8.6	66.4	0.0	33.6

Table 3.1. Time course of peak over time in the Patuxent River experimental and control incubations. Peaks are grouped percentages of total peaks within the three molecular weight ranges: MW > BSA (100-1,000 kDa), MW < BSA (2-52 kDa), or MW BSA (within the range of 52-100 kDa)

When molecular weight peaks in the control incubations were grouped into the same categories as described above, many differences were observed from the experimental incubation (Table 3.1). The peaks in replicate 1 were used as a comparison, because there was little bacterial contamination in the incubation (see Table 3.2). There were no small, modified products observed, except at 0 hours and 12 hours with 5.8% and 18.9% of the total proteinaceous material observed, respectively. At the beginning of the incubation, 9.7% of the total proteinaceous material is present in the high molecular weight fraction. At 6 hours the high molecular weight fraction increased to 53% and then remained relatively constant although highly variable throughout the incubation. Although the molecular weight

Experiment Treatment			Control Treatment		
Time (hrs)	Replicate	Bacteria cells ml $^{-1}$ (10^{6})	Time (hrs)	Replicate	Bacteria cells ml $^{-1}$ (10^{3})
0	1	6.87	0	S-1	7.47
0	2	11.1	0	S-2	107
0	3	15	0	S-3	4.98
24	3	2.33	92	S-3	0
48	3	2.56	92	US-1	0
92	1	1.25	92	US-2	22.9
92	2	2.48	92	US-3	5.85
92	3	2.08			

Table 3.2 Bacterial abundances (cells ml^{-1}) in the Patuxent River incubation for selected replicate samples. S-1 through S-3 indicates sampled control incubation replicates, while US denotes the unsampled control incubation.

peak corresponding to BSA was variable over the course of the incubation, it remained relatively high from the beginning of the incubation (84.5%) to 48 hours (54.6%), and then decreased at 92 hours (33.6%).

3.2.2 Bacterial Abundances in River Incubations

Replicate natural water incubations showed variable protein concentration results, particularly early in the incubations. Much of this variation might be reflected in differences in bacterial counts, which may suggest differences and thus variability in bacterial growth between the replicates (Table 3.2). Replicate 1 in the experimental incubations started with the lowest bacterial abundance (6.87×10^6 cells mL^{-1}) as well as the highest BSA concentration (3.35 μg mL^{-1}). Replicates 2 and 3 were more similar in bacterial abundance with 11×10^7 cells mL^{-1} and 15×10^7 cells mL^{-1}, respectively. Replicates 2 and 3 were also more similar in BSA concentrations at the start of the experiment, with concentrations of 1.17 μg mL^{-1} and 1.09 μg mL^{-1} (Figure 3.6). All experimental replicates showed slight declines in bacterial abundances over the time course of the experiment (Table 3.2).

The control incubations had a significantly lower bacterial count than the experimental incubations (Table 3.2), with replicate 2 showing the highest bacterial contamination (1.07×10^4 cells mL^{-1}) from early in the experiment. In replicates 1 and 3 the bacterial abundance started low, with an abundance of 7.47×10^3 and 4.98×10^3, respectively. Only the bacterial abundance of replicate 3 was measured at 92 hours and there was no bacteria present in the water sample. With the knowledge that

a low number of bacteria were present at the start of replicate 3, the loss of bacteria at the end of the incubation would indicate the presence of grazers.

In the unsampled control incubations, replicates 2 and 3 had a normal range of bacteria (2.3×10^6 cells mL^{-1} and 5×10^5 cells mL^{-1}, respectively), meaning that the filtration did not remove all the bacteria from the sample (Table 3.1). Replicate 1 showed expected results with bacteria abundance at 92 hours negligible. Interestingly, replicate 1, which contained few bacterial at the end of the incubation, was only slightly higher in BSA concentration (1.2 ug mL^{-1}) than the other two contaminated replicates (Figure 3.6), which both had concentrations around 0.9 ug mL^{-1}.

3.3.3 Comparisons of Protein Degradation among Environments

The Patuxent River, lower Delaware Bay, marsh, and Chesapeake Bay incubations all showed a rapid decrease in BSA concentration from 0 to 24 hours (Figure 3.7). In the marsh incubation BSA was not detected after 24 hours. In the Chesapeake Bay and lower Delaware Bay incubations, BSA was absent by 36 hours. In the Patuxent River there was a significant decrease in BSA concentration throughout the experiment, with small amounts detected throughout the incubation period.

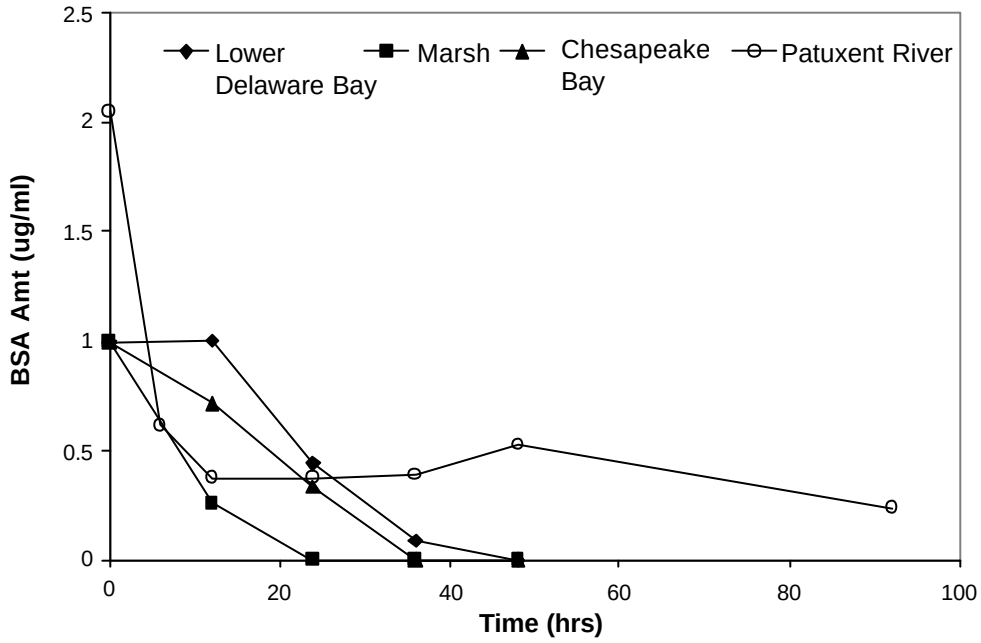


Figure 3.7 Time course of dissolved BSA concentration in four different natural water environments.

Examination of size-exclusion separations from these incubations demonstrates that although different sized products are formed, the 3 sites showed the same quick disappearance of BSA. Modified products appeared as rapidly as seen in the Patuxent River incubation. Yet there were some differences in the chromatograms between the 4 sites (Figure 3.8).

In the Chesapeake Bay incubation we observed lower molecular weight modified products formed at 24 hours (Figure 3.8a). There was a range of products from 20 kDa to 300 kDa. The peaks at 24 hours in the Chesapeake Bay incubation were much more defined than the other sites, suggesting that there were fewer low molecular weight intermediates formed in this incubation. There was also a significant void volume peak (>1000 kDa) at 12 hours that remained at the same concentration throughout the incubation. This peak suggests that aggregation of

proteinaceous material occurred in the incubation, which was also observed by (Otte, Lomholt et al. 1997). By 48 hours there is only the void volume peak seen by fluorescence.

In the lower Delaware Bay site, the degradation of BSA occurred more rapidly than the other sites (Figure 3.8b). Additional modified products were observed at 12 hours in this particular incubation. These peaks surrounded BSA and ranged from 10 kDa to 1000 kDa in size. The modified products remained in the incubation through 24 hours. There was a significant decrease in proteinaceous material by 36 hours seen by fluorescence, but the high molecular weight peak remained throughout the incubation. Smaller modified products (5-10 kDa) were observed throughout the incubation. By 48 hours, only the largest molecular weight peak remained in the incubation.

The freshwater marsh incubation differed significantly from the other sites (Figure 3.8c). Both larger and smaller molecular weight products were observed at

12 hours ranging from approximately 17 kDa to 500 kDa. There was also a significant peak which coeluted in the void volume (>1000 kDa) that existed at the same intensity through 36 hours. All products other than the void volume disappeared from marsh waters by 24 hours, which would likely indicate rapid utilization of low molecular weight material and rapid aggregation of material to form high molecular weight material. The >1000 kDa peak was not visible in the incubation at 48 hours, unlike any of the other natural water incubations examined. This could indicate rapid adsorption of all proteinaceous material to container walls, or rapid utilization of the full molecular weight range of proteinaceous material

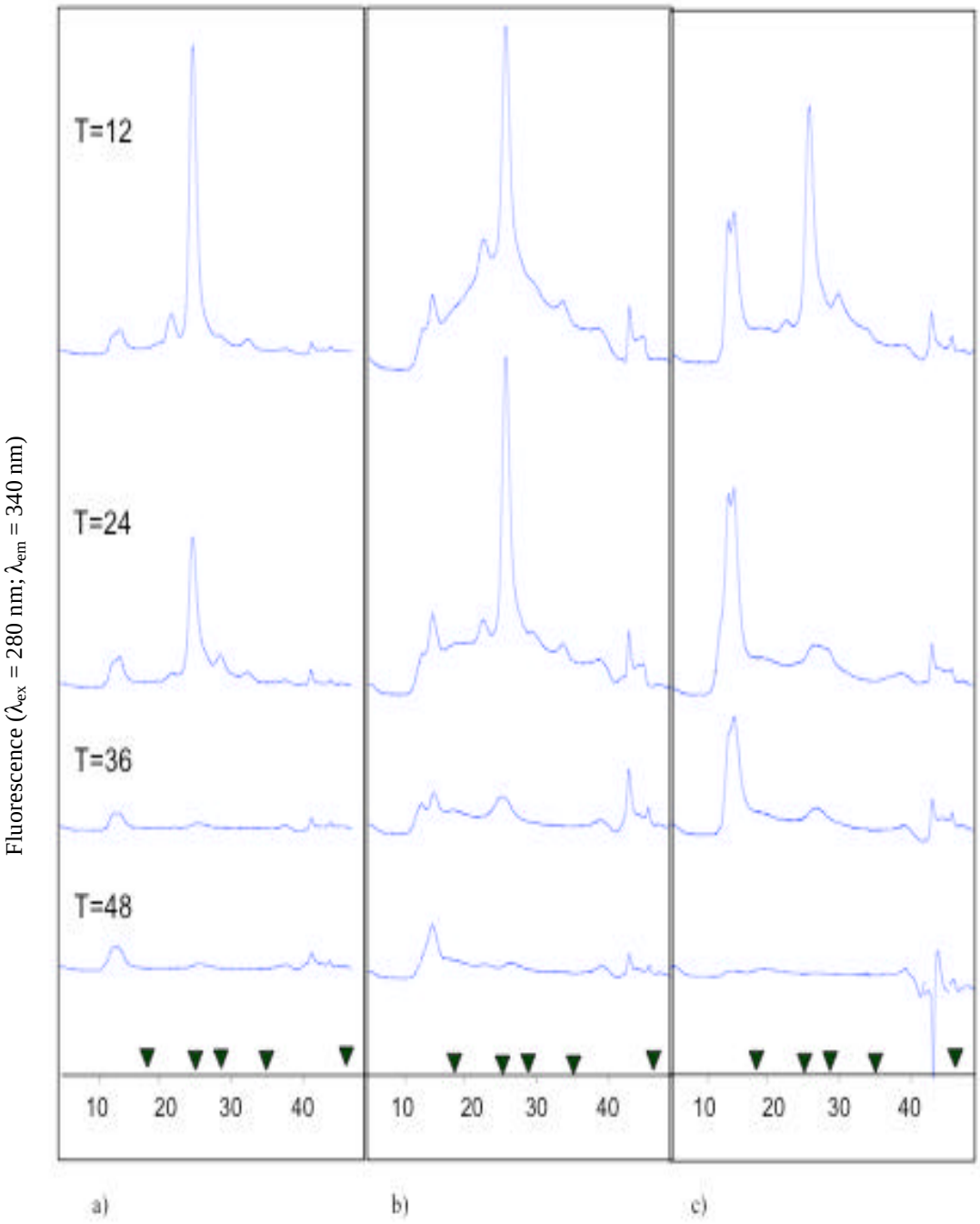


Figure 3.8 Size-exclusion chromatograms of protein modification and degradation from 3 different aquatic environments: a) Chesapeake Bay b) lower Delaware Bay and c) marsh. Along the x-axis is elution time in minutes.

produced by 48 hours in the marsh sample. In all the incubations, peaks of interest were collected for further examination by amino acid analysis and exact molecular weight determination by LC/MS.

3.3.4 Amino acid analysis

Figure 3.14 illustrates the total amino acid mole percent distributions over the time course of the incubations at three sites (anoxic site- Chesapeake Bay, open estuary- lower Delaware Bay site, and marsh site). To compare past dissolved combined amino acid results with our data, we first needed to be sure the extraction and concentration processes used did not change amino acid compositions. Results show that there are no changes in BSA amino acid composition before and after concentration with an Ultrafree centrifugal filter (Figure 3.9). The yield of our amino acid hydrolysis method is 85-90% of total BSA. The variance of the amino acid composition is approximately ± 2.3 % as seen by multiple BSA standard runs.

The total amino acid concentrations show similar trends to the dissolved protein concentrations found by size-exclusion chromatography results, verifying our protein quantification method. The total hydrolyzable amino acids (THAA) of the fraction we analyzed (5 kDa to 0.45 μm) in each incubation (Chesapeake Bay, lower Delaware Bay, and marsh) showed slightly different trends over the time of the incubation (Table 3.3). Interestingly, the Chesapeake Bay incubation showed a relatively large decrease in amino acids over time, while the lower Delaware Bay

showed a relatively small decrease in total amino acid concentration, and the marsh incubation remained relatively constant throughout the experiment (Figure 3.10).

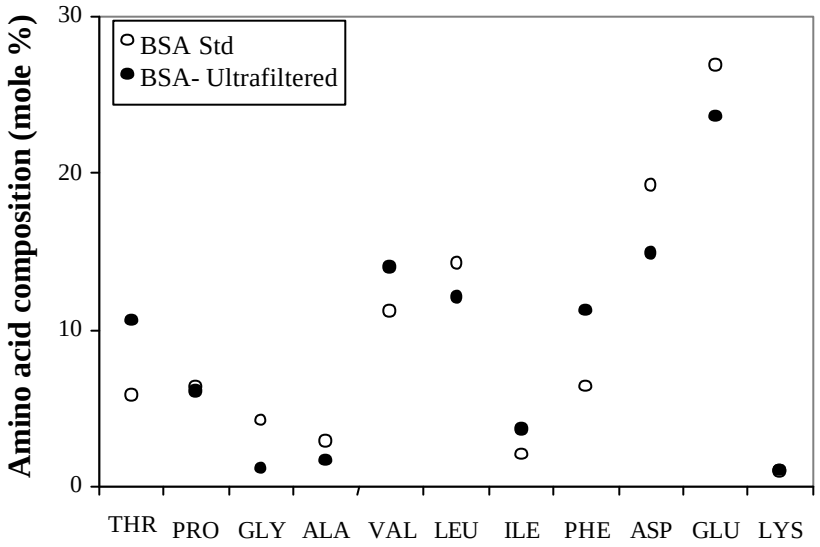


Figure 3.9. Amino acid composition of BSA standard compared to BSA after ultrafiltrafiltration Amino acids are abbreviated as follows: Threonine (THR), proline (PRO), glycine (GLY), alanine (ALA), valine (VAL), leucine (LEU), isoleucine (ILE), phenylalanine (PHE), aspartic acid and asparagines (ASP), glutamic acid and glutamine (GLU), and lysine (LYS).

		Marsh Incubation				Chesapeake Bay Incubation				Lower Delaware Bay Incubation				Glucose
		Time (hrs)												
		12	24	36	48	12	24	36	48	12	24	36	48	24
Uncharged, Polar	Thr	0.03	0.14	0.19	0.16	0.24	0.13	0.00	0.08	0.03	0.06	0.02	0.02	0.07
Nonpolar	Pro	0.04	0.06	0.27	0.11	0.48	0.10	0.14	0.36	0.06	0.13	0.02	0.02	0.02
	Gly	0.06	0.38	0.08	0.98	0.23	0.16	0.34	0.25	0.06	0.23	0.09	0.19	0.08
	Ala	0.18	0.18	0.08	0.62	0.29	0.42	0.32	0.28	0.36	0.32	0.09	0.08	0.47
	Val	0.08	0.10	0.12	0.12	0.47	0.07	0.05	0.11	0.09	0.16	0.01	0.03	0.25
	Leu	0.05	0.14	0.04	0.17	0.75	0.31	0.14	0.20	0.20	0.31	0.02	0.06	0.12
	Ile	0.05	0.03	0.13	0.04	0.14	0.03	0.09	0.07	0.03	0.04	0.01	0.02	0.02
	Phe	0.09	0.08	0.24	0.12	0.16	0.19	0.07	0.06	0.14	0.23	0.03	0.04	0.06
Charged, Polar	Asp	0.08	0.48	0.94	0.55	3.44	0.62	0.48	0.49	0.36	1.02	0.15	0.11	0.40
	Glu	1.17	0.07	0.86	0.14	0.82	0.14	0.94	0.09	0.16	0.35	0.04	0.04	0.05
	Lys	0.00	0.02	0.63	0.05	0.58	0.05	0.30	0.04	0.01	0.08	0.04	0.01	0.06

Table 3.3 Amino acid concentrations (nM) of total hydrolysable amino acids(THAA) in the size range of 5 kDa to 0.45 um during protein incubations. Control (Glucose) addition is included for blank comparison.

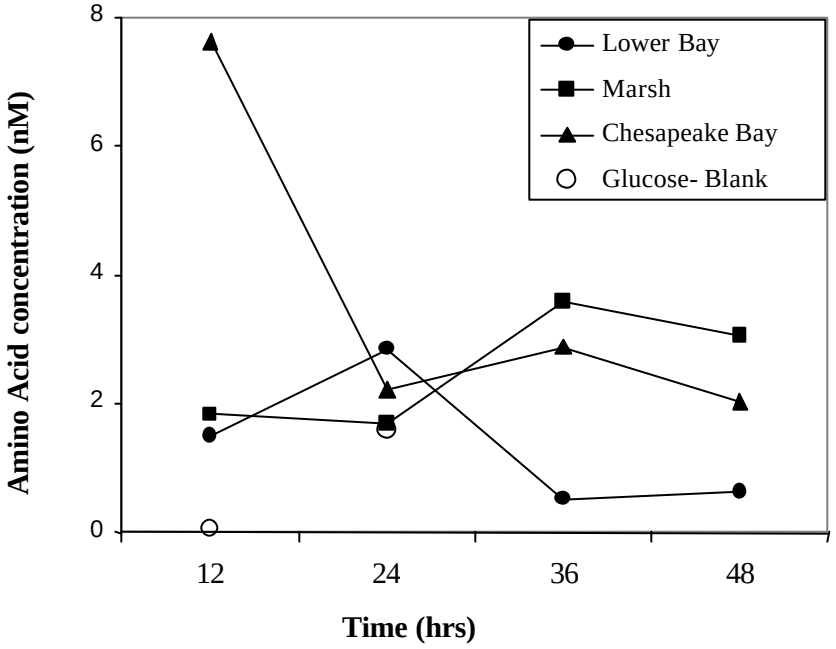


Figure 3.10 Time course of amino acid concentration during 3 different environment incubations. Total amino acid concentrations include glucose additions as a blank.

Whole Samples

Table 3.4 shows individual amino acid composition shifts over time, however for simplicity, amino acids were grouped by side chain functionality to examine the potential for selective preservation. The BSA standard contains 41.7% charged, polar amino acids, 51% nonpolar amino acids, and 7.1% uncharged, polar amino acids (Figure 3.11). At the start of the marsh incubation, the bulk of the amino acids were made up by the charged, polar amino acids (68.6%), followed by nonpolar amino acids (29.9%), then uncharged, polar amino acids (1.5%) (Figure 3.11a). At the end of the experiment the amino acid distribution shifted to 24.1%, 70.7% and 5.2%, respectively. Overall there was an increase in nonpolar amino acids, a decrease

	Marsh Time (hrs)				Chesapeake Bay Time (hrs)				Lower Delaware Bay Time (hrs)			
	12	24	36	48	12	24	36	48	12	24	36	48
THR	1.51	8.37	5.37	5.18	3.14	5.81	0.03	3.92	1.91	2.09	3.96	3.78
PRO	2.15	3.69	7.45	3.53	6.29	4.43	4.78	17.94	3.75	4.40	3.46	3.58
GLY	3.46	22.75	2.28	32.04	3.08	7.15	11.76	12.10	4.14	7.75	17.67	29.50
ALA	9.68	10.78	2.20	20.28	3.82	18.89	11.26	13.93	24.02	10.89	16.73	12.17
VAL	4.57	5.76	3.26	4.03	6.20	3.03	1.64	5.55	6.14	5.56	2.85	5.55
LEU	2.81	8.01	1.01	5.46	9.86	14.25	4.77	10.01	13.28	10.70	3.10	9.63
ILE	2.51	1.81	3.60	1.35	1.90	1.20	3.20	3.31	1.78	1.51	1.38	2.56
PHE	4.71	4.94	6.82	4.00	2.06	8.72	2.41	2.80	9.49	7.92	6.00	6.91
ASP	4.15	28.43	26.33	17.95	45.21	27.99	16.88	24.01	23.88	34.64	29.67	17.48
GLU	64.45	4.23	24.11	4.69	10.82	6.43	32.77	4.64	10.73	11.94	8.20	6.69
LYS	0.00	1.23	17.58	1.47	7.63	2.10	10.49	1.81	0.87	2.59	6.98	2.15

Table 3.4 Individual amino acid composition in mole percent of whole dissolved protein samples (5 kDa-0.45 um fraction) collected from 3 separate incubations (marsh, Chesapeake Bay, and Lower Delaware Bay) at the specified time points.

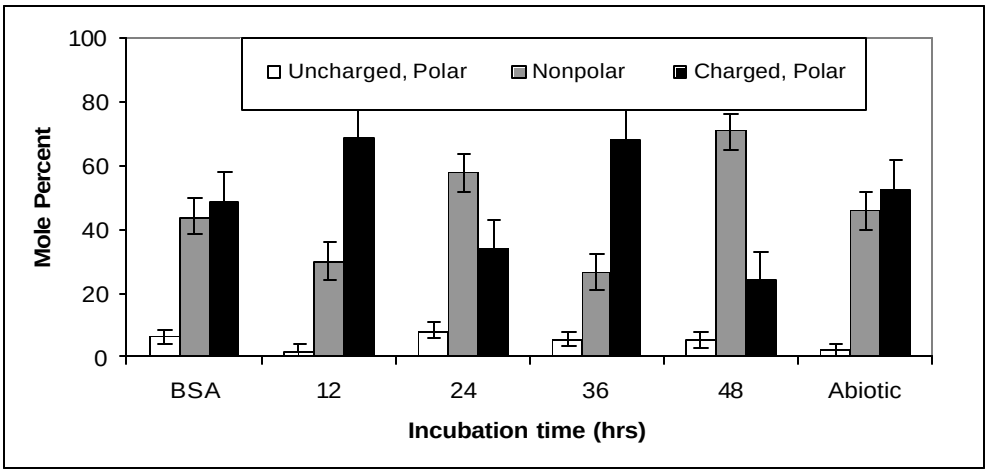
in amino acids with polar, charged side chains, and the uncharged, polar amino acids remain unchanged. For the nonpolar amino acids, glycine and alanine increased

significantly during the incubation, while valine, phenylalanine, leucine, proline, and isoleucine remained unchanged. It is important to note that although leucine did not change through the incubation, it was found to be lower than the BSA standard. The decrease in amino acids with the polar charged side chain was mainly due to a large decrease in aspartic acid, lysine remained largely unchanged, while glutamic acid was quite variable throughout the incubation (Figure 3.11a).

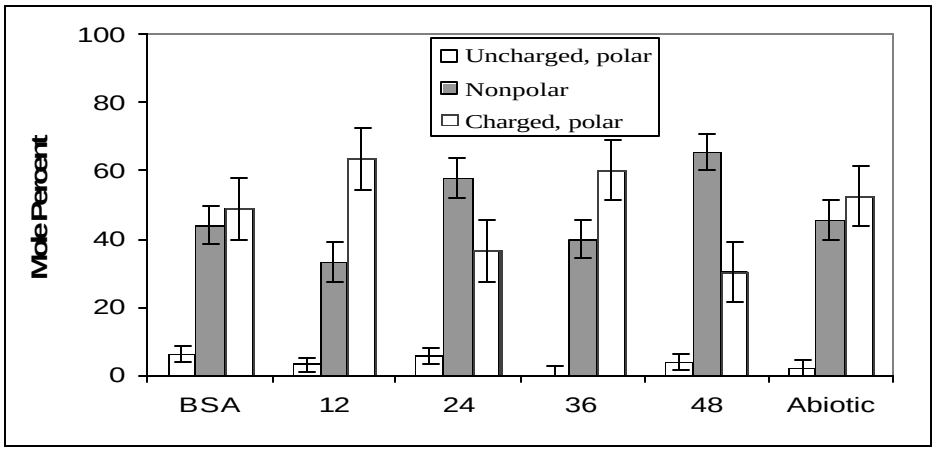
The Chesapeake Bay incubation showed similar trends of amino acid distribution as the marsh incubation (Figure 3.11b). At the start of the incubation, the bulk of the amino acids were made up by the charged, polar amino acids (63.7%), followed by nonpolar amino acids (33.2%), then uncharged, polar amino acids (3.1%). At the end of the experiment the amino acid distribution shifted to 30.5%, 65.6%, and 3.9%, for charged, polar amino acids, nonpolar amino acids, and uncharged polar amino acids, respectively. Overall there was an increase in nonpolar amino acids, a decrease in amino acids with polar, charged side chains, and the uncharged, polar amino acids showed no significant changes. For the nonpolar amino acids, glycine and alanine increased significantly during the incubation, while phenylalanine, leucine, valine, proline, and isoleucine remained unchanged. All amino acids with polar charged side chains decreased through the incubation and overall they were significantly lower than the BSA standard (Figure 3.11b).

In contrast, the lower Delaware Bay differed from incubations at other sites (Figure 3.12c). The dominant amino acids at the beginning of the experiment were the nonpolar amino acids (62.6%), followed by the amino acids with polar charged side chains (35.5%), and uncharged, polar amino acids (1.9%). This composition did

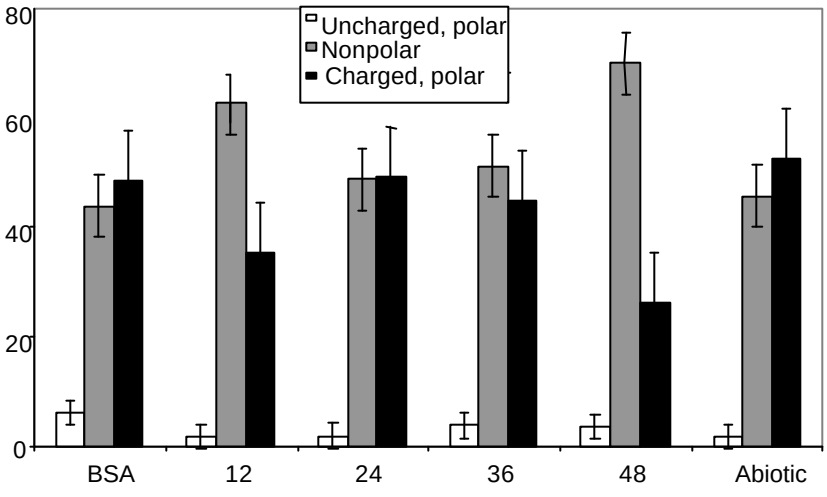
not shift significantly to the end of the incubation with a distribution of 69.9%, 26.3%, and 3.78%, respectively. Among the amino acids with nonpolar side chains, glycine showed a significant increase while leucine and alanine showed a significant decrease in the incubation, and valine, isoleucine, proline, and phenylalanine remained largely unchanged. Interestingly, glycine and alanine were higher in composition than the BSA standard and leucine was lower. For the polar, charged amino acids, aspartic acid showed a significant decrease over time, while lysine increased over the incubation, and, unlike the other incubations, glutamic acid remained relatively unchanged. Although lysine increased through the incubation, it was significantly lower than the BSA standard at all time points.



a)



b)



c)

Figure 3.11 Amino acid mole percent distribution of whole fraction samples in a) marsh, b) Chesapeake Bay, and c) lower Delaware Bay at time 12 hours, 24 hours, 36 hours, and 48 hours compared to a BSA standard and an abiotic control.

Size-exclusion Peaks of Interest

In addition to comparing whole sample amino acid composition and concentrations, specific peaks were collected by size-exclusion chromatography and analyzed individually. The general observation from past results is that amino acid compositions remain largely unchanged throughout protein degradation (Siezen and Mague 1978; Henrichs and Farrington 1987; Nguyen and Harvey 1994; Nguyen and Harvey 1997). To test this hypothesis we examined whole samples as well as individual molecular weights to understand what molecular weight range causes a shift in amino acid composition. If BSA remained the dominant peak, it would be assumed that the amino acid distribution would be very similar to BSA. If lower molecular weight intermediates or high molecular weight products were formed, did these peaks dominate the amino acid distribution?

To simplify the following results, the amino acids have again been grouped by side chain functionality. Also, the size-exclusion peaks were also grouped as molecular weight > BSA (100-1000 kDa) and molecular weight < BSA (2-52 kDa), and compared to BSA (52-100 kDa) in amino acid composition (Table 3.5; Figures 3.12-3.14). In the marsh incubation at 12 hours (Table 3.5), the dominant group was the amino acids with the charged, polar side chains (50.9%) of THAA in the >BSA fraction, followed by nonpolar amino acids (43.5%), and then the uncharged, polar amino acids (5.6%). This distribution is very similar to the BSA standard of 51.0%, 41.7%, and 7.1%, respectively. The molecular weight fraction of <BSA of the marsh incubation at 12 and 24 hours, showed a very different trend from BSA with amino acid distribution of 16.2%, 77.7%, and 6.6%, respectively at 24 hours. From the

individual amino acid composition shifts (Figure 3.12) it can be seen that as the incubation progresses, the individual amino acid composition shifts back toward the original BSA composition. It is important to note that even the peak found at 66 kDa, equivalent to BSA, showed minor compositional changes from the BSA standard composition, with charged, polar amino acids, nonpolar amino acids, and uncharged, polar amino acids at 42.3%, 53.6%, and 4.1%, respectively, including an increase in glycine, lysine, and glutamic acid, and a decrease in valine.

	Chesapeake Bay incubation					Lower Delaware Bay incubation					Marsh incubation					
	24 hr 45 kDa	24 hr 29 kDa	24 hr 17 kDa	48 hr 65 kDa	48 hr 12 kDa	24 hr 165 kDa	24 hr 66 kDa	24 hr 13 kDa	36 hr 66 kDa	48 hr 66 kDa	12 hr >1000 kDa	12 hr 165 kDa	12 hr 66 kDa	12 hr 44 kDa	12 hr 7 kDa	24 hr 64 kDa
THR	2.18	1.35	3.66	21.86	21.63	3.04	2.92	3.39	21.63	16.28	6.43	1.15	4.11	3.44	6.17	15.17
PRO	5.59	3.68	3.14	24.81	4.21	2.93	2.67	6.90	4.21	17.96	5.58	4.83	3.59	5.50	2.08	18.06
GLY	26.85	21.20	18.88	28.12	29.37	16.19	24.16	26.71	29.37	32.22	2.35	32.22	18.25	18.73	33.53	6.79
ALA	17.35	13.84	19.53	5.19	6.24	10.16	13.52	15.23	6.24	8.97	6.51	4.80	12.63	19.48	20.69	3.84
VAL	27.71	4.37	5.51	0.83	14.56	6.34	4.88	4.13	14.56	10.00	6.79	4.59	6.43	8.48	21.46	16.52
LEU	4.59	4.92	11.33	10.09	4.70	5.84	5.17	5.03	4.70	6.40	10.78	6.51	4.82	4.60	1.97	9.69
ILE	3.39	1.72	4.26	4.19	4.35	3.25	2.18	3.25	4.35	3.90	4.33	1.21	1.83	1.70	1.98	5.31
PHE	2.49	2.94	5.22	1.04	1.87	2.94	2.12	2.28	1.87	1.63	4.46	4.50	6.05	2.21	1.37	4.98
ASP	3.02	18.98	12.64	0.97	2.09	13.32	11.91	13.67	2.09	0.23	15.98	14.67	8.93	11.34	5.37	1.18
GLU	4.17	19.29	12.11	1.15	4.85	24.02	19.73	16.13	4.85	2.03	31.57	20.65	20.37	16.30	2.47	6.50
LYS	2.66	7.71	3.73	1.76	6.14	11.97	10.75	3.29	6.14	0.38	5.22	4.87	13.00	8.22	2.89	11.96

Table 3.5 Amino acid composition percent distribution of total proteinaceous material peaks collected from size exclusion chromatography of natural water incubation samples. All peaks within the size ranges of LMW 92-52 kDa, BSA 52-100 kDa, and HMW 100-1000 kDa were combined and the percent of the total amino acids observed within each group was calculated. The marsh incubation contained a combination of 12 and 24 hour peaks. The Chesapeake Bay contained 24 and 48 hour peaks, which were separated for a closer examination as indicated, and the lower Delaware Bay contained 24, 36, and 48 hour peaks collected.

In the Chesapeake Bay incubation (Table 3.5), no peaks of molecular weight greater than BSA were collected. However, peaks <BSA were collected at 24 hours and 48 hours. At 24 hours the dominant amino acid group was the nonpolar amino acids (67.4%), followed by the charged, polar group (29.5%), and then the uncharged polar amino acids (3.1%). At 48 hours this distribution shifted to 68.6%, 9.7%, and

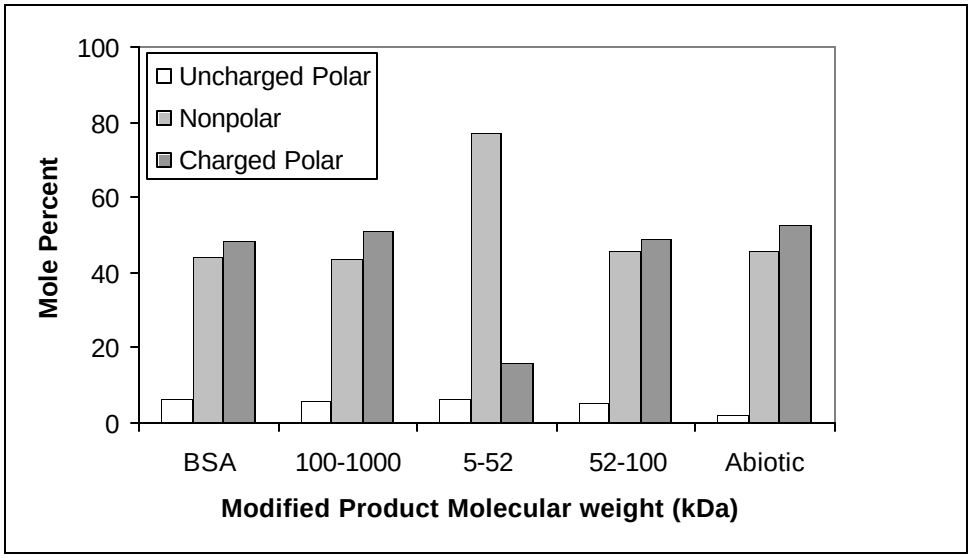


Figure 3.12 Amino acid composition in mole percent of selected peaks at named time points in the marsh incubation. Molecular weight ranges of amino acid mole percent groups.

21.7%, respectively. The nonpolar amino acid group remained relatively unchanged, while the polar charged group decreased, and the uncharged polar group increased over time. It is important to note that the low molecular weight fraction at 48 hours was unique in that there is a significant increase in the uncharged, polar amino acids. This was the first occurrence of a high percentage seen for this amino acid group (the rest were below 10%). However, a look at the individual amino acid composition

shifts for the selected peaks (Figure 3.13) shows that as the incubation progresses, overall the individual amino acid composition shifts back toward the original BSA composition as in the marsh incubation.

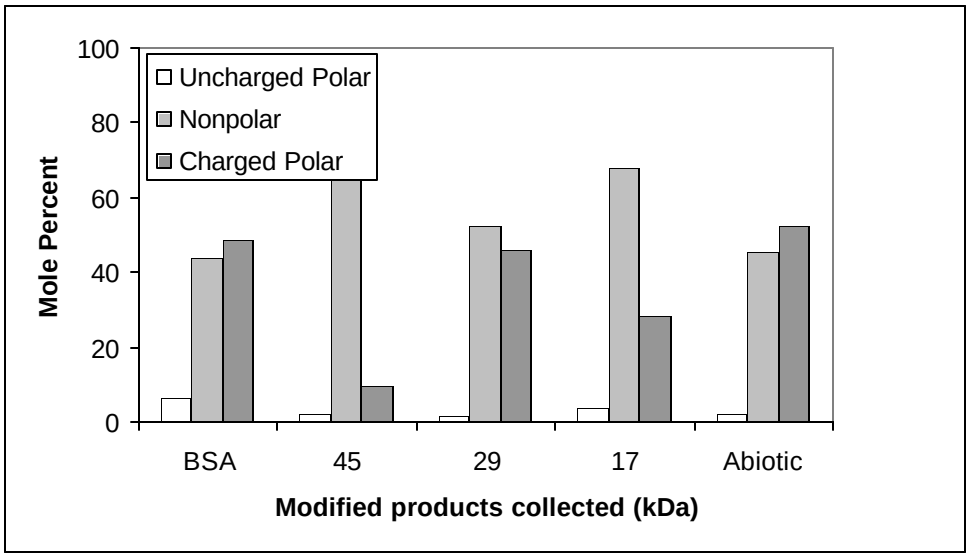


Figure 3.13 Amino acid composition in mole percent of selected peaks at named time points in the Chesapeake Bay incubation. Comparison of the 5-52 kDa modified products of the BSA incubation at 24 hours and compared to BSA and an abiotic sample at 92 hours.

In the lower Delaware Bay incubation (Table 3.5, Fig. 3.14), the >BSA molecular weight fraction collected at 12 hours was similar to that seen in the marsh incubation. The composition was similar to BSA (51.0%, 41.7%, and 7.1%, for charged, polar; nonpolar; and uncharged, polar amino acids, respectively), with charged polar amino acids (49.3%) as the dominant group, followed by nonpolar amino acids (47.7%), and uncharged, polar amino acids (3.0%). The <BSA fraction was similar to the marsh

incubation, with a distribution of 33.1%, 63.6%, and 3.4% for charged polar, nonpolar and uncharged polar, respectively. In this incubation, the 66 kDa peak

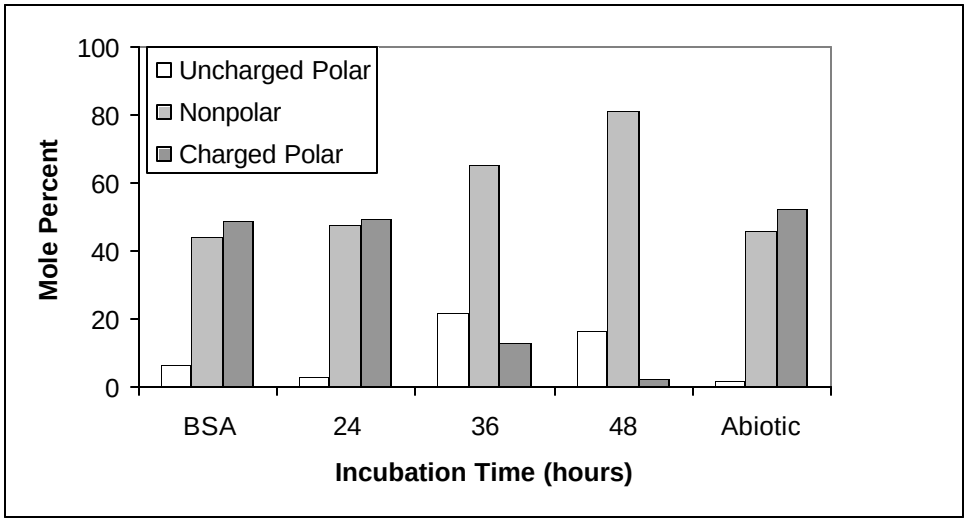


Figure 3.14 Amino acid composition in mole percent of selected peaks at named time points in the lower Delaware Bay incubations. Comparison of the 52-100 kDa peak at the 3 indicated incubation times. They are compared to the BSA standard as well as an abiotic sample taken at 92 hours.

corresponding to BSA was collected at 24, 36, and 48 hours. The distribution of amino acids in this peak was different than expected for BSA, with nonpolar amino acids dominating (74.9%), followed by uncharged, polar amino acids (17.1%), and then charged polar amino acids (7.7%). The uncharged polar amino acids were about 10% higher than in the standard and the charged, polar amino acids about 30% lower than in the standard. In the 36 hour and 48 hour samples, the 66 kDa peaks were the driving force of increasing the uncharged, polar amino acids as seen in the whole samples. A look at the individual amino acid composition of the collected peaks did not show a shift over time towards the original BSA composition as seen in the other

two incubations, but instead showed an overall further increase in the charged, polar amino acids, and a further decrease in nonpolar amino acids.

3.3.5 ESI-MS analysis

The Patuxent River water incubation was chosen for further analysis by electrospray ionization mass spectrometry (see figure 3.6b for the size-exclusion chromatograms of this incubation). This form of molecular weight analysis is much more sensitive than size-exclusion chromatography, being able to measure accurate protein mass.

Most intermediate products were observed up to 24 hours into the incubations, and after this point, protein concentrations were too low for analysis. Results showed a shift to lower molecular weight immediately upon the addition of the model protein, BSA, to 66.2 kDa corresponding to a loss of approximately 3 amino acids (Figure 3.15). A BSA standard gives a molecular weight of approximately 66.4 kDa. Smaller intermediate products were observed at the beginning of the incubation (64.8 kDa) corresponding to a loss of approximately 11 amino acids. Intermediate products were in the same range with slight shifts in molecular weights at 12 hours (66.4 equaling BSA and 63.3 kDa, -20 amino acids). At 12 hours additional smaller intermediate product was observed, considerably smaller than BSA at 45.1 kDa (-142 amino acids). There were also slight shifts in molecular weights from BSA (66.3, -1 amino acid and 57.7 kDa, -58 amino acids). At 24 hours, the largest peak observed had a molecular weight of 57.9 kDa (-57 amino acids). Interestingly, the peak at 45.1

kDa observed at 12 hours, remained at 24 hours with about the same peak intensity, indicating that it was unused once produced. Please note that the size-exclusion chromatograms are not to scale and the peak intensities are greatly reduced by 24 hours, so minor products are more obvious as seen in Figure 3.6.

Section 3.4 Discussion

3.4.1 Protein recovery

There are many procedural hurdles to dissolved protein analysis in aquatic environments. These issues include low natural dissolved protein concentration below normal detection on most analytical equipment commonly used in protein research, complexation with organic matter, and interferences by ions found in natural waters. These issues make it necessary to include an extraction, concentration, and isolation step prior to analysis. With extra steps inherently comes decreases in yields, and therefore, the method must be optimized in order to overcome low beginning concentrations for further analysis.

A number of studies describe the concentration and isolation of dissolved proteins in natural waters (see Chapter 2 for review). Ultrafiltration appears to be the most effective in concentrating and desalting natural water samples. Centrifugal ultrafiltration devices were chosen because they are more time efficient than large ultrafiltration devices for the small volumes in the present research. (Nguyen and Harvey 1994) developed a method for the quantification of particulate protein in

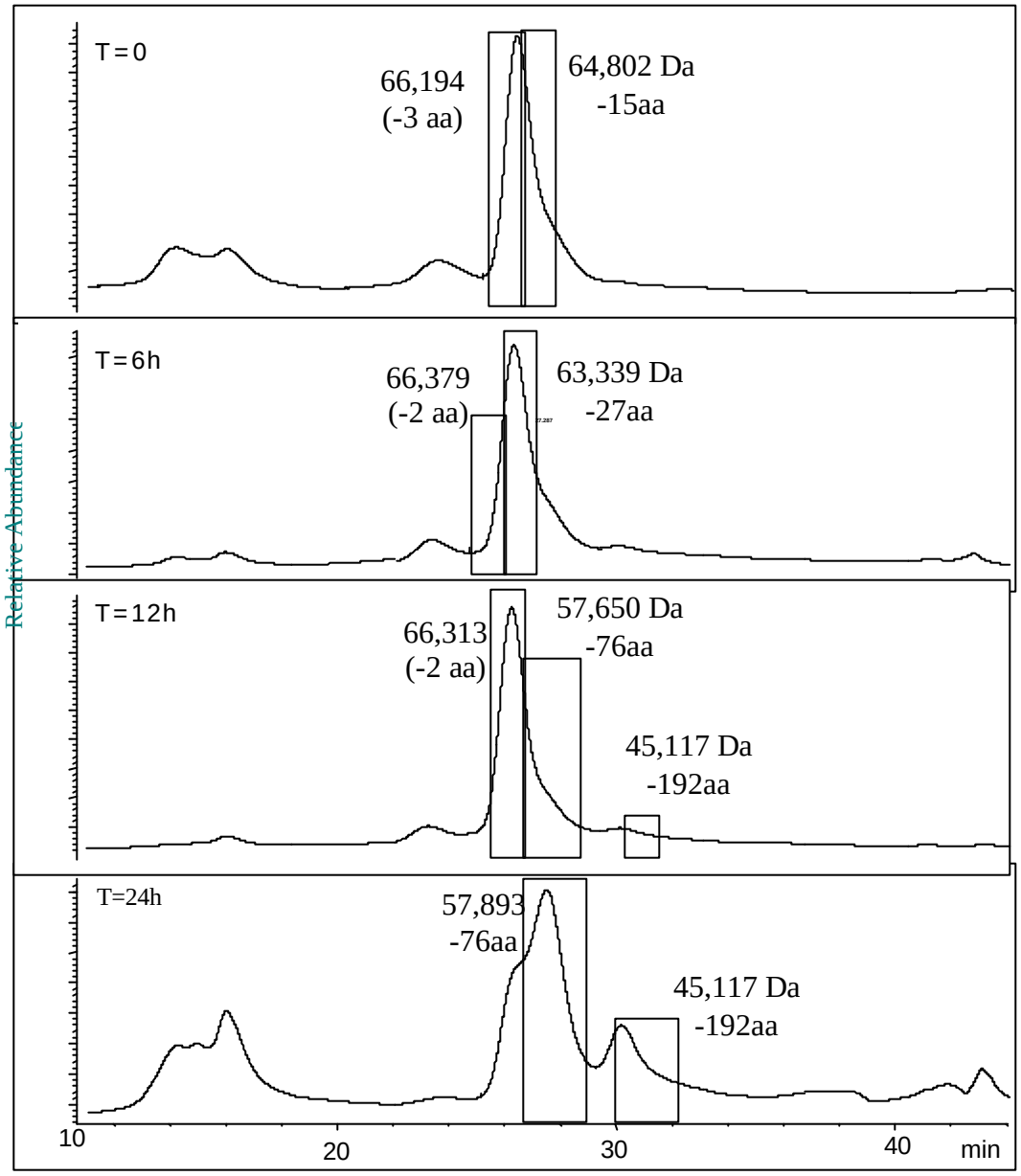


Figure 3.15 The time course of the Patuxent River incubation as depicted by size-exclusion chromatography. The shaded boxes indicate the peaks collected and further analyzed by electrospray ionization (ESI) mass spectrometry. The numbers next to the boxes indicate first the mass of the peak (in Da) calculated using Agilent deconvolution software after ESI-MS. The second number indicates how many amino acids would be lost from the original protein to achieve the calculated mass.

aquatic systems using the bicinchoninic acid assay. This method is not as accurate for dissolve proteins (described in Chapter 2). Measuring particulate proteins in natural water samples requires the samples to first be filtered to remove salts and small molecules which act as known interferences, such as free sugars (Smith, Krohn et al. 1985). These small interferences cannot be easily separated from dissolved protein by filtration due to the similarity in size and properties. A more accurate method for quantification of dissolved proteins in aquatic systems is the use of size-exclusion chromatography with fluorescence detection. There are no known interferences with this quantification method, and the instrument can be used for both quantification and separation for further analysis.

Adsorption may be a factor in the loss of dissolved protein from our laboratory incubations. Following low levels of radiolabeled BSA, Hollibaugh and Azam (1983) found that 23-30 % of all protein adsorbs to container within minutes. The use of larger containers to decrease the surface to volume ratio would help to minimize the adsorption issues. The Patuxent River incubations were performed in 1 L bottles, whereas the other 3 incubations were performed in 20 L carboys. Assuming that protein adsorption occurs within minutes, we would expect to see rapid adsorption to container walls in the Patuxent River incubation.

In the present study, 20-70% of the total BSA added to the incubation was recovered after sample processing. However, 93% of the total BSA added was recovered after simple concentration with centrifugal filters, without being incubated first. Within the size fraction analyzed (5 kDa- 0.45 um), we estimate that 30% of the protein is sorbed to container walls (we assume this is constant in each incubation),

decreasing the total yield. If we remove this loss from the yield, we can assume that the actual yield is 60-100%, which is more acceptable for this method and confirms previous results by Hollibaugh and Azam (1983). It appears that a major source of loss of protein in the protein degradation experiments is adsorption to the container walls and other organic matter with time. For simplicity of further analysis, it is assumed that the loss by adsorption is constant throughout the incubation. However, it is possible that over time protein may be removed from the walls of the incubation container and utilized by the bacteria present.

The utilization of adsorbed protein by bacteria is poorly understood. Some research has suggested that adsorbed proteins are degraded more rapidly that dissolved proteins at the same concentrations (Taylor 1995). This increased utilization may be due to an increased concentration near a surface, and possibly partial denaturation of the protein due to interactions between the substrate and internal hydrophobic surfaces (Soderquist and Walton 1980; Taylor 1995). Other findings contradict these results, showing that adsorbed proteins actually are utilized more slowly (Samuelsson and Kirchman 1990; Nagata and Kirchman 1996). Taylor (1995) observed that the type of protein and substrate can be important factors in sorption of protein. In some cases, adsorbed proteins may rapidly degrade due to concentration and subsequent denaturation between the substratum and internal hydrophobic surfaces, exposing an increased number of bonds for hydrolysis (Soderquist and Walton 1980; Taylor 1995). Because there is not an increase in BSA or lower molecular weight material over the course of the experiment, it suggests that the adsorbed protein is not being rapidly modified or degraded in these incubations.

3.4.2 Modification and degradation of protein as seen by SEC

Size-exclusion chromatography proved to be a superior method to the colorimetric assays presently available for the quantification of dissolved proteins extracted from natural waters. It allows for the quantification of specific molecular weight ranges over the time course of an incubation to follow modification and degradation. This method also permits the collection of intermediate products for further analysis. Unfortunately, the molecular weight resolution of the column and flow rates used in the present study limits determination to a 0.5 minute wide peak, which is equivalent to 25 kDa. Therefore, molecular weight estimates for collected peaks incorporates a range of molecular weights from the apex of the peak (specified) ± 12.5 kDa.

Many studies have been performed to further understand kinetic rates of bacterial utilization of proteins and amino acids, and, consequently protein degradation (Hollibaugh and Azam 1983; Kirchman and Hodson 1984; Kirchman 1990; Keil and Kirchman 1991; Keil and Kirchman 1993). Even so, specific proteins preserved have not been identified except in one case (Tanoue papers) and the enzymatic mechanisms for utilization and preservation are not well understood. As expected, protein degradation is rapid in natural water incubations. This is in agreement with past data showing that bacterial degradation of BSA is rapid, with BSA depletion by over half within the first 6 hours and most removed by 12 hours in all experimental replicates (Hollibaugh and Azam 1983). However, in the present study the formation of new intermediate products were observed. During protein degradation, Hollibaugh and Azam (1983) did not observe intermediate modified

products from the starting material of BSA. However, the present observations are consistent with more recent results showing that intermediate products of protein degradation can be observed during a short time course protein degradation as seen in natural water incubations (Keil and Kirchman 1991; Keil and Kirchman 1993; Pantoja and Lee. 1994).

While investigating the formation of new peaks, a broadening of the original peak found at 67 kDa (identified as BSA) was observed over the time course of the incubations. This indicates that multiple molecular weight products are formed very early in the degradative process which appear similar in size to the original protein, BSA. These products appear to be related to BSA by sequential loss of amino acids. Our results also suggest that as the BSA is sequentially hydrolyzed, the intermediates are released back into the environment. These intermediates are then available for further degradation. These results are consistent with recent work in sediments suggesting that not all intermediate products formed from protein degradation are immediately taken up, but instead some were reverted back to the water (Nunn, Norbeck et al. 2003). Vetter et al (1998) suggested a similar model where proteins in sediments are partially hydrolyzed and the leftover intermediate product is then released. In this way, peptide intermediates could be transferred between bacterial groups. This contribution can help to understand the transfer of high molecular weight DOM to low molecular weight DOM. With the release of modified products, there is more chance of chemical modifications as indicated by increases in molecular weight over the time course of the incubations.

The enzymatic mechanisms of protein degradation in natural waters are important in determining rates and patterns of dissolved protein degradation. Several models exist that describe the bacterial degradation of protein, but the widely accepted model suggests that extracellular enzymes are released to hydrolyze proteins, and the products are either taken in by bacteria or remain in the environment (Mayer, Schick et al. 1995). The small molecular weight changes observed in the protein incubations agree, indicative of the action of an exoprotease, which hyrolyze amino acids from the ends of proteins (Vetter, Jumars et al. 1998). This is different from the process of endoproteases, which clip a protein at a specific peptide bond, leading to the cleavage of a protein into small molecular weight pieces (Vetter, Jumars et al. 1998). In the present study, these lower molecular weight peaks could be investigated further to examine amino acid compositional shifts (See section 3.4. amino acids). Amino acid analysis can give a better understanding of which amino acids are lost or preserved from the model protein or if protein degradation is an entirely random process.

Table 3.1, shows that while BSA was degraded rapidly through the experimental incubation, high molecular weight products were also being formed. High molecular weight material is formed during protein incubations, similar to previously documented results (Nguyen and Harvey 1998; Nguyen and Harvey 2001; Nguyen and Harvey 2003) during protein degradation. The present data assist in challenging the long held belief that HMW material is unlikely to survive except in the presence of minerals and inorganic matrices. The increase of high molecular

weight intermediate products from 0 to 24 hours can possibly be explained by complexation with organic matter.

Macromolecular complexation by microbes and hydrophobic and hydrogen bond interactions has been suggested as a mechanism of preservation (Lee and Henrichs 1993; Nguyen and Harvey 2001). Keil and Kirchman (1993) found that proteinaceous material might undergo abiotic chemical modifications, such as Schiff-base reactions, described as condensation with sugar, leading to insolubility and resistance to enzymatic attack. In this theory, extracellular enzymes would bind to proteins that have undergone a chemical modification, leading to a large refractory molecule (Lee and Henrichs 1993).

This theory could also explain why the bicinchoninic acid assay did not provide a reproducible was measure of dissolved protein concentration. It is known that macromolecules can interfere with the accuracy of the assay (Smith, Krohn et al. 1985). In support of adsorption and abiotic complexation, the control incubation showed similar trends. There was a 50% decrease in BSA from 0-6 hours, but there was a 50% increase in >1000 kDa molecular weight material in this same time frame. It is clear that aggregation took place in this time period in both the control and experimental treatments.

We therefore propose a process where larger molecular weight material is formed that is more refractory than the original dissolved protein. It is possible that this high molecular weight material may be less able to bind easily to container walls and therefore are more easily detected by fluorescence throughout the incubation. Such high molecular weight material increases through the incubations suggest that

dissolved protein and modified material are complexing over time. In the unsampled control, a higher BSA recovery was observed in all replicates and a lower abundance of high molecular weight products. These results may indicate that exposure to air assists in adsorption and aggregation. There was lower aggregation and lower loss in the unsampled control compared to the sampled control. It is unknown what causes this increase in recovery and prevents aggregation.

Bacterial counts decreased slowly during the time course of the experimental incubation. This suggests either than substrate was limiting or more likely that grazers were present in the incubation keeping the bacterial population from growing as expected. Grazers were also observed in the marsh and lower Delaware Bay incubations based on organic marker profiles (Harvey et al., in prep). The sampled control replicate 3 contained bacteria at the beginning of the experiment and lacked bacteria at the end of the incubation also suggests that the sample may have contained grazers which depleted the bacterial population.

Comparison of bacterial utilization between environments

The four environments were chosen for their different seawater properties and potential for differing bacterial community structure. The choices include a freshwater marsh site, open estuary site (lower Delaware Bay), anoxic site (Chesapeake Bay), and a river site (Patuxent River). The marsh site is high in humics and terrestrial organic matter, possibly allowing for higher preservation rates through adsorption and other abiotic preservation processes. The open estuary, anoxic site,

and Patuxent River are all lower in organic matter content and contain different bacterial communities.

There was not much difference in protein degradation between the different incubations. All of the incubations showed a rapid decrease in BSA, indicating that the existing bacterial community does not have an effect on relative degradation rates. In parallel work, Harvey et al. (in prep), found that the addition of BSA to natural water incubations at each site, showed no consistent shifts in bacterial communities based on 16S RNA phylogenetic analysis (FISH). Cottrell and Kirchman (2000) showed similar results when examining the utilization of DOM and simultaneous bacterial community shifts in estuarine and coastal environments in that utilization differed by the bacteria present and did not correlate with bacterial group abundance shifts. This data along with the present observations suggests that the composition of the bacterial community does not strongly alter protein degradation patterns. There is probably a mix of bacteria present in all communities to utilize all existing proteins and products formed.

The formation of high molecular weight products (Figure 3.6b and 3.8) was slightly different in the four environments. If in fact dissolved protein complexes with organic matter, creating more refractory, high molecular weight material, we would assume that the marsh incubation would have the highest aggregation rates, then Patuxent River, followed by the Chesapeake Bay and lower Delaware Bay with the lowest organic content. In agreement with this hypothesis, the marsh had the highest percentage of high molecular weight proteinaceous material than any other environment. The high molecular weight composition (41%) by 12 hours in the

Patuxent River suggests rapid protein complexation. The high molecular weight proteinaceous material in the lower Delaware Bay remained low (approximately 30%) until 48 hours. The observations from this incubation and the Chesapeake Bay were as expected if the high molecular weight observed during the present protein incubations is due to complexation of protein with organic matter. From these results, we can suggest that the organic matter content of the environments may influence the degree of high molecular weight proteinaceous material and, therefore, the degree of protein preservation.

3.4.3 Amino acid analysis

Sources of organic nitrogen in the environment include proteins, polypeptides, peptides, free amino acids, nucleic acids, amino sugars, chitin, and lipid N. Lipid N and chitin were the only nitrogenous compounds not found to be extremely labile. We know mineralization is efficient, but a small portion of high molecular weight material can escapes early diagenesis and be preserved. Keil and Kirchman (1991) found that bacteria preferentially use dissolved free amino acids over dissolved combined amino acids, unless amino acid concentration in the environment are low. Bacteria use dissolved free amino acids as a source of C, N, and energy and therefore it usually is found only in low concentration through uptake and release (Williams, Berman et al. 1976). In contrast, dissolved combined amino acids are utilized 2-10 times slower than dissolved free amino acids (Azam, Smith et al. 1992) and are the largest identified component of dissolved organic nitrogen (Keil and Kirchman 1993).

It is important to consider the mechanisms of protein utilizations to understand the presence of dissolved combined amino acids in aquatic systems.

Bovine serum albumin has a very different amino acid composition than many aquatic proteins (Nguyen and Harvey 1994). This is important in the present research because we can observe changes in the BSA amino acid composition without believing that it may be confused with another protein. The amino acid composition shifts were similar in the marsh and the Chesapeake Bay. The results show that over time the modified products begin to look more similar in amino acid composition to BSA. This indicates that there is selective attack on the protein, first removing charged polar amino acids, causing an indicated preservation of nonpolar amino acids, specifically glycine and alanine. When investigating using mole percentage, it is important to note that a decrease in one amino acid always corresponds to an increase in another. Although the natural conformation of BSA is not known, it can be speculated that these charged polar amino acids occupy the outer shell of BSA when it is in its natural conformation. After these amino acids are removed and the interior of the protein is exposed, remaining amino acids are utilized equally, with no selection. This data is supported by the peaks collected around BSA over time, suggesting that charged polar amino acids are depleted and there is a corresponding increase in glycine, indicating preservation. It is also supported by Aguilar and al. (1998) showing that when trypsin was added to adsorbed proteins, the hydrophobic domains of the protein remain intact, indicating that these regions were protected by adsorption. The hydrophilic portions were exposed to the enzyme, and therefore attacked.

The higher molecular weight intermediates (>100 kDa) observed were similar in amino acid composition to BSA. This material was in high abundance and when hydrolyzed would produce the most amino acids. Therefore it can be expected that when total amino acids are analyzed, no changes in amino acid compositions would be observed. These results show that it is necessary to look at individual intermediate processes to fully understand the mechanisms involved in the breakdown of BSA. This material may contain significant amounts of native BSA or that with minor caused by small changes in the chemical properties of BSA through degradation.

The present research differs somewhat from past results showing that there is no selective attack on amino acids in protein decay (Rosenfeld 1979; Wakeham, Lee et al. 1984; Henrichs and Farrington 1987; Nguyen and Harvey 1997). Preservation of glycine and serine as seen in the present results, has been previously observed, but was attributed to the refractory nature of diatom cell walls (Hecky, Mopper et al. 1973; Siezen and Mague 1978; Lee and Cronin 1984). Carlson, Mayer et al. (1985) suggested that binding of certain amino acids to macromolecular organic matter may be a mechanism for glycine enrichment.

It is interesting to note that different phytoplankton have all been found to have very similar amino acid compositions (Cowie and Hedges 1992; Nguyen and Harvey 1994). Dissolved combined amino acids in various waters all had very similar amino acid compositions, including the Delaware estuary (Keil and Kirchman 1993), particulate matter from surface oceanic and coastal waters (Siezen and Mague 1978), Peru coastal upwelling waters (Wakeham, Lee et al. 1984), and particulate organic matter in Arctic and Antarctic waters (Hubberten, Lara et al. 1995).

Although similar amino acid compositions exist in most environments due to similarity in amino acid protein composition, attack by bacteria may first require selective attack of external amino acids, namely the charged, polar amino acids. Once the interior of the proteins is exposed, all amino acids are used at equal rates.

In the lower Delaware Bay the amino acid composition trends were not similar to the Chesapeake Bay and the marsh site. The samples analyzed provided a more detailed examination of what occurred to the products within 52-100 kDa range, because a this peak was collected at each time point. Over time there was an increased loss in charged polar amino acids, and increased preservation of nonpolar amino acids, specifically glycine and alanine. This data implies that there is selective attack on the charged amino acids, while the bacteria are selectively leaving the nonpolar amino acids behind. Unlike the other two incubations, over time there was not a shift in the amino acid composition towards the original BSA composition, possibly indicating a slower rate of exposure of the interior nonpolar amino acids of BSA, and therefore a continued selective attack. Some amino acids compositions (leucine, isoleucine, phenylalanine) remain the same throughout the incubations, which indicates that there is no selective utilization of these proteins.

3.4.4 ESI/MS analysis

Electrospray ionization mass spectrometry, which can detect changes down to 100s of Da, is considerably more sensitive at distinguishing molecular weight changes through the degradation of protein than size-exclusion chromatography,

which can detect changes in molecular weight of 10 kDa. This can be an invaluable tool in the further understanding of protein degradation and the contribution of protein to organic matter cycling. Proteins and peptides could be analyzed intact, without hydrolysis to amino acids components.

The ability of electrospray ionization MS to detect whole protein molecular weight is limited by signal stability and the mass to charge range of the ion trap. Formic acid is added to increase the charges on the protein, which increases the intensity of the signal. The model protein, BSA, used in this research was not the best protein choice for electrospray ionization mass spectrometry as a stable signal cannot be obtained due to the chemical characteristics of the protein and the mass-to-charge distribution of BSA is nearing the maximum range of the ion trap. In future research it would be useful to test the use of smaller proteins as models for dissolved protein degradation in natural waters.

Results of the electrospray ionization of the Patuxent River incubation confirmed that protein degradation occurs rapidly in the presence of natural microbial communities. The molecular weight changes observed are consistent with (Pantoja and Lee 1999; Nunn, Norbeck et al. 2003), who found that 2 to 3 amino acids are hydrolyzed from a protein during degradation, then the protein product leftover is released into the environment for further attack. It is unknown where these amino acids are hydrolyzed; however it is suggested that they are hydrolyzed from the ends of the protein (Panjota and Lee 1999; Nunn, Norbeck et al. 2003). An examination of the primary structure of BSA (Hirayama 1990), shows that nonpolar amino acids are situated on the terminal ends of the protein. If the ends of the protein are being

hydrolyzed first, these nonpolar amino acids would be preferentially lost. Our results of ESI-MS, in combination with our amino acid analysis, show instead a loss in charged polar amino acids was observed. Although the actual natural conformation of BSA is not known, it can be assumed from our research and the fact that BSA is considered a soluble protein that hydrophilic, charged polar amino acids would be situated on the exposed face of the dissolved protein and the hydrophobic amino acids in the center. This would suggest that as the hydrophilic amino acids are used the protein products would become more hydrophobic than the original protein. From Nguyen and Harvey (2001), it can be seen that hydrophobic interactions increase during degradation and lead to aggregations. So as modifications to BSA make the protein more hydrophobic, there is an increase in aggregation. This result is consistent with our findings of high complexation of proteinaceous material in all incubations examined. It would be an important advance to determine if these modified products actually originate from BSA using LC/MS peptide mapping tools.

Future analysis might include peptide mapping to determine where the amino acids are being lost from, to confirm our suggestions that they are being removed from the exposed face of the protein as opposed to the terminal ends of BSA. There are limitations in that the entire tertiary structure of the protein has not been identified, but protein origin can often be determined from peptide mapping. Further improvement of the LC/MS method includes a choice of a more appropriate model protein to optimize the signal stability of the instrument. A peptide mapping tool capable of accurately identifying a modified protein and the changes occurring to them would be invaluable to this line of research.

3.5 Conclusions

Although proteins are degraded rapidly in natural waters, intermediates can be observed over short time periods. The use of size-exclusion chromatography can give a view of what intermediate products are formed by fluorescence detection. A collection of these intermediate products and further amino acid analysis shows that charged, polar amino acids are lost from BSA and nonpolar amino acids are preserved. Over the time course of the incubations, the amino acids compositions shifted to more closely resemble the amino acid composition of the original protein. After careful examination of the primary structure of BSA, we can propose that the protein is not being hydrolyzed from the ends, but instead that the charged, polar amino acids are situated on the outside of the protein in its natural conformation. As these amino acids are lost from the protein, the interior amino acids are exposed and all amino acids are utilized at the same rate, meaning there is no selective preservation once the interior of the protein is exposed. Further examination is needed to identify amino acid location in natural conformation of BSA and peptide mapping to detail where on BSA hydrolysis is occurring and verify that the modified products observed originated from BSA.

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