

Beyond the Mineral Sorptive Protection Hypothesis: Protein Preservation in Marine Systems via Formation of High- Molecular-Mass Aggregates

The principal source of organic matter in marine systems is derived from in situ primary production. After the crash of a phytoplankton bloom, much of this organic matter is remineralized in the water column and sediments, but a small fraction survives diagenesis to be preserved. Of particular importance to global carbon and nitrogen budgets is the inherent resistance of organic matter to decay, or its transformation into biologically refractory substances, which removes it from the active cycles in the oceans. Little is known, however, about the chemical composition of this refractory, macromolecular organic matter or mechanisms contributing to its resistance during diagenesis.

Since proteins and their constituent amino acids are the most abundant nitrogen-containing substances in phytoplankton, they are potentially important sources of carbon but more importantly nitrogen in marine systems. Proteins serve as important substrates for bacterial growth, for the most part being rapidly recycled in the marine water column¹. Historically, proteins have been considered very labile, and consequently unlikely to survive as high-molecular-mass components during early diagenesis. Evidence is increasing, however, that some fraction of "proteinaceous" material is preserved in the aquatic environment². While the recent work of Pantoja and Lee revealed that high-molecular-mass proteinaceous material comprised a significant fraction of the nitrogen in some sediments, many questions remain as to whether proteins are selectively preserved or modified².

Although the concept of sorptive preservation can help to explain much of the distribution of organic carbon in oceanic, much controversy currently remains over mechanisms that retain proteinaceous material in the geosphere. Sorption to clay minerals has been shown to inhibit bacterial degradation of proteins. However, we have evidence from previous work that proteinaceous material can survive in systems where minerals are absent or only present in low amounts^{1,3,4}. These recent results raise questions about additional mechanisms for preservation.

Hypothesis:

Preservation/formation of high-molecular-mass proteinaceous aggregates

Based on Nguyen and Harvey's study⁵, protein incubated with proteases were performed to assess whether the extracted high-molecular mass aggregates remaining after extensive degradation could be receptive to enzymatic attack. Extended incubations with trypsin showed that much of the aggregates are subject to proteolytic cleavage (Figure 1). These results are direct evidence for the preservation of peptide linkages in sediments as old as 4000 years. Amino acid analysis of the aggregates indicated the presence of numerous trypsin cleavage sites, specifically arginine and lysine residues (Table 1). Assuming the aggregates have an average molecular mass of 1,500,000, we might predict a total of 13,000 amino acid residues in each aggregate, of which ~500 to 700 are potential cleavage sites. The extent of digestion is not only dependent on amino acid composition but also on the accessibility of arginine and lysine residues within the macromolecule. The major plant enzyme RuBisCo has about as many arginine and lysine residues (~500) as the high-molecular mass aggregates from diatom detritus and surficial

sediments, but only it is susceptible to digestion to all lower molecular mass peptides (Table 1). These results suggest that tightly associated proteins are preferentially preserved.

Size-exclusion chromatograms under native and denaturing conditions point to noncovalent associations as a major mechanism for the preservation of proteinaceous material. The shift in molecular mass of extracted proteins treated with various denaturants such as guanidine, organic solvents, or detergents indicates the highly hydrophobic nature of the surviving proteinaceous material (Figure 1). While RuBisCo is completely denatured with guanidine (Figure 1A), the proteinaceous aggregates in algae, water column detritus, and sediments are not. These denaturation studies again suggest that strongly associated proteins are preserved.

Nguyen and Harvey⁵ have three hypotheses for the aggregates observed in algae, detritus, and sediments. (1) Aggregates may be large protein complexes such as Clp proteases, plant enzymes that may form heteromeric complexes of up to 1700 kDa. These proteases are found in phytoplankton and might survive diagenesis. Other multi-subunit proteins normally found in algae are additional possibilities. (2) Aggregates represent proteins that have unfolded after cellular death and membrane disruption. In cells, proteins are normally found in their native conformation as monomers, or as multimers containing several polypeptides. Proteins are stabilized to a large degree by hydrophobic forces. Unfolding results in the exposure of hydrophobic polypeptide regions, which may result in various inter- and intramolecular associations or aggregations. This hypothesis is based on observations of protein aggregations by biochemists for pure protein solutions after relatively short-term storage. (3) Aggregates include hydrophobic proteins (e.g., lipases, membrane proteins) that are only partially soluble in the aqueous buffers and therefore would “clump” together in solution.

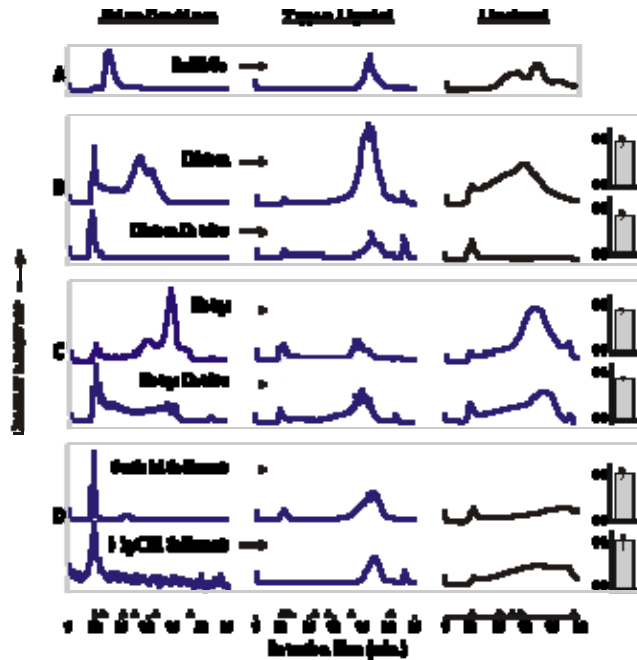


Figure 1. Size-exclusion chromatograms of proteinaceous material separated under native conditions, in the presence of the endoproteinase trypsin, and under guanidine-denaturing conditions. Samples include (A) the plant enzyme RuBisCo; (B) proteins from diatom cells and detritus; (C) proteins from green algal (*Botryococcus*) cells and detritus; and (D) proteins from surficial and 10-m deep (4000-year old) sediments of Mangrove Lake, Bermuda. Relative molecular mass markers (triangles) from left to right on each time scale: 1.5×10^6 , 6.7×10^5 , 1.6×10^5 , 4.4×10^4 , 1.7×10^4 , 1.3×10^3 . RuBisCo is shown as a control. Bar graphs show the fraction of total nitrogen attributed to protein-N. Protein consistently comprises 40 to 60% of total nitrogen in the algal, detrital, and sediment samples⁽⁵⁾.

Protein	Total No. of Amino Acids	No. of Arginines	No. of Lysines	Arginines & Lysines	% digested
RuBisCo	4,768	264	264	528	100
aggregates, diatom detritus	~13,000	195	290	485	92
aggregates,	~13,000	300	230	530	72

surficial sediments					
aggregates, 9.7 m deep sediments	~13,000	130	585	715	98

Table 1. Total number of amino acids, the potential number of trypsin cleavage sites (after arginine and lysine residues), and the digestibility of RuBisCo and high-molecular-mass proteinaceous aggregates(5).

In general, folded proteins are normally resistant to proteases. Local unfolding probably precedes proteolytic cleavage of the polypeptide chain. Thus, the stability of a protein would be expected to determine its rate of degradation. The digestion conditions with trypsin were performed at 37°C, which might have been high enough to induce partial unfolding of the aggregates, allowing for initiation of cleavage. Under natural conditions of marine systems, proteolytic enzymes likely would not be as efficient.

Encapsulation of proteins by aliphatic cell wall and membrane components

In order to understand how differences in cellular matrices can influence organic matter diagenesis, Nguyen and Harvey¹ studied the kinetics of decay of three diverse algal species, including a marine diatom (*T. weissflogii*), a cyanobacterium (*Synechococcus* sp.) and a dinoflagellate (*P. minimum*). Despite the vastly different cell wall matrices of the algae, protein from these species degraded at identical rates (Figure 2). We have recently studied the degradation of the green alga *Botryococcus* under identical conditions, knowing that this alga produces a highly refractory macromolecule surrounding a typical cellulosic cell wall⁵. Our results show that the protein fraction from this green alga is lost about 8 times slower than protein from the other algae (Figure 2).

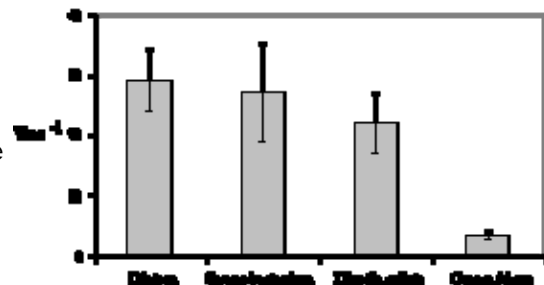


Figure 2. First-order rate constants (\pm 95% CL) for the oxic degradation of protein from four algal species(5).

Based on current results, we also hypothesize that some of the high-molecular mass aggregates may be associations of protein with membrane and/or cell wall components. A study by Laursen et al.⁶ suggests that detritus may be selectively enriched in cell membrane fragments with associated proteins. Another study indicates that partially denatured proteins are more likely to associate with liposomal lipid bilayers. These associations could result in SEC yielding relative molecular masses that are much higher than the proteins' actual molecular mass.

The organic matter monolayer on minerals

Chemisorptive attachment to mineral surfaces, in general, may be an important process for the preservation of labile organic matter in sediments and has been put forth as one factor which controls organic matter concentrations in marine sediments. Studies investigating sorption of soluble organic matter to mineral surfaces indicate the following: (1) there is a relationship between organic matter accumulation and mineral surface area; (2) adsorption to mineral surfaces is reversible for a significant fraction of organic matter; and (3) the number of organic matter coatings varies with concentration. Unfortunately, we still do not have a good handle on the nature of organic matter binding to minerals. Figure 3 is a conceptual diagram illustrating the different types of protein associations that might occur on mineral surfaces.



Figure 3. Conceptual diagram illustrating a potential range of proteinaceous states on mineral surfaces or within mineral pores.

This diagram is based on past studies of protein and bulk organic matter sorption, and on our recent work. Hypothetically speaking, for a monolayer-equivalent loading, the sorption process may involve a complete unfolding of proteins⁷. We believe that protein preservation cannot be explained solely by sorption. Protein-protein aggregations and encapsulation within aliphatic cellular material are hypothesized to play important roles in preservation in organic-rich sediments.

Research Objectives: This study is designed to elucidate the mechanisms of protein preservation in particulate matter and coastal sediments, and to characterize the primary (sequence) and three-dimensional structure of the proteins. Characterization of algal cells and detritus generated in the laboratory will help to link water column processes with sedimentary processes. In addition, we will take a mechanistic approach to determine if we can observe the alteration of pure proteins on various mineral surfaces. New analytical approaches including the use of several ultrahigh-resolution mass spectrometers will help to address the following hypotheses. (1) Phytoplankton-derived polypeptides can resist microbial degradation in the water column and sediments by strong noncovalent aggregations and that these intermolecular polypeptide associations are largely determined by conformational alterations (or stability) and the hydrophobicity of the original polypeptides during diagenesis. (2) Encapsulation of proteinaceous material into refractory biopolymers (e.g., lipid bilayer fragments or cell wall) also plays an important role in preservation in diverse coastal sediments. Our objectives will be addressed using proteins in controlled laboratory experiments to examine the changes in structure and the factors that influence the alterations. We will also evaluate how these conformational alterations affect proteolytic activity and ultimately the retention of specific polypeptide sequences. In addition, we propose to conduct a comprehensive molecular analysis of proteinaceous material extracted from several coastal sedimentary environments.

References:

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³ Knicker, H.; Hatcher, P. G. Survival of protein in an organic-rich sediment: Possible protection by encapsulation in organic matter. *Naturwissenschaften*, 1997, 84, 231-234.

⁴ Nguyen, R. T.; Harvey, H. R. Protein preservation during early diagenesis in marine waters and sediments. *Nitrogen-containing Macromolecules in the Bio- and Geosphere*. ACS Symposium Series 707, 1998, pp. 88-112.

⁵ Nguyen, R. T.; Harvey, H. R. Preservation of protein in marine systems: Hydrophobic and other noncovalent associations as major stabilizing forces, *Geochimica et Cosmochimica Acta*, 2001, 65(9), 1467-1480.

⁶ Laursen, A. K.; Mayer, L. M.; Townsend, D. W. Lability of proteinaceous material in estuarine seston and subcellular fractions of phytoplankton. *Mar. Ecol. Prog. Ser.*, 1996, 136, 227-234.

⁷ Taylor, G. T. Microbial degradation of sorbed and dissolved protein in seawater. *Limnol. Oceanogr.*, 1995, 40, 875-885.