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Size distribution and amino acid chemistry of base-extractable proteins from Washington coast sediments

BROOK L. NUNN* and RICHARD G. KEIL
*School of Oceanography University of Washington, Seattle, WA 98103, USA; *Author for correspondence (e-mail: brookh@u.washington.edu)*

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Abstract. Proteinaceous components from four Washington coast margin sediments were extracted with base, fractionated into one of four size classes (< 3 kDa, 3–10 kDa, 10–100 kDa, > 100 kDa), and analyzed for their amino acid contents. Base-extracted material accounts for ~30% of the total hydrolyzable amino acids (THAA) and each size fraction has a unique composition, regardless of where the sediment was collected (shelf or upper slope). The < 3 kDa size fraction (~10% of base-extractable THAA) is relatively enriched in glycine (~30 mol%), lysine (~5 mol%), and non-protein amino acids (~5 mol%). Glycine and non-protein amino acids are common degradation products, and lysine is very surface active. We suggest that the < 3 kDa size fraction, therefore, represents a diagenetic mixture of fragments produced during the degradation of larger proteins. The 3–10 and 10–100 kDa size fractions (~10% and 42% of base-extractable THAA, respectively) have similar amino acid distributions dominated by aspartic acid (~ 30 mol%). Enrichments in Asp is likely due to both preservation of Asp-rich proteins and the production of Asp during degradation. The > 100 kDa size fraction (~38% of base-extractable THAA) is not dominated by any particular amino acid and can not be modeled by mixing the amino acid compositions of the other size fractions. We propose that the larger size fractions (10–100 kDa and > 100 kDa) represent intact, or near intact, proteins. Estimates of isoelectric points and relative hydrophobicity suggest the base-extractable proteins are primarily acidic and have globular structures. Statistical comparisons to several known proteins indicates that the base-extractable component is most similar to planktonic cytoplasmic proteins.

Introduction

Sediments contain a mixture of proteins and peptides at various states of degradation (Nguyen and Harvey 1999; Pantoja and Lee 1999; Keil et al. 2000). This heterogeneous assortment is typically extracted in bulk and hydrolyzed to amino acids using hydrochloric acid (total hydrolyzable amino acids: THAA). The method destroys information about protein chemistry, providing only amino acid compositions. For certain sample types (e.g. clastic marine sediments) an amino acid-based degradation index (Dauwe et al. 1999a) can be used to roughly evaluate the degradation status of hydrolyzable

amino acids. However, because hydrolysis results in the loss of sequence information, the interplay between protein or peptide type and the reactivity of this proteinaceous material in environmental systems remains largely unexplored (Nguyen and Harvey 2001). Previous attempts to develop methods for extracting proteins intact from sediments have met with limited success. Nuygen and Harvey (1994) developed a technique for organic-rich materials, but the method is ineffective in mineral-laden systems (Nunn and Keil, submitted). Currently, the best available method for extracting proteins and peptides from sediments without hydrolyzing them is to use base (Nunn and Keil, submitted).

If proteins and peptides could be quantitatively extracted, then powerful tools could be brought to bear on unanswered questions regarding protein source and stability in the environment. For example, Ostrom et al. (2000) used mass spectral techniques to confirm the preservation of intact osteocalcin in 53ky old bone fragments and Nunn et al. (2003) used mass spectral techniques to determine the bacterial hydrolysis patterns of proteins added to marine sediments and waters. Currently, widespread application of these techniques to natural marine samples is limited by the inability to quantitatively isolate, purify and identify individual protein components from the heterogeneous environmental matrix (Nunn 2004).

One alternative to mass spectral determinations of protein size or structure that currently can be applied is to extract the proteinaceous fraction non-destructively and isolate different size classes prior to acid hydrolysis and amino acid quantification. Understanding the size and chemistry of these different fractions can provide information about the types of proteins or peptides that are present in environmental samples. In this manuscript we examine the amino acid chemistry of different size fractions of base-soluble material isolated from four marine sediments from the Washington margin. While the extracts comprise only 23–31% of the total THAA, they none-the-less represent the quantitatively largest component of the nitrogen-containing pool of organic matter isolated from marine sediments to date. The proteinaceous materials from the four sediments have similar size distributions and amino acid compositions. Amino acid compositions traditionally associated with degradation and diagenesis are only present in the smallest size fractions (< 3 kDa). The larger size fractions have amino acid compositions diagnostic of intact proteins. Thus, there are differences in the extractable pool of proteins and peptides that are associated with size. This suggests a size-reactivity spectrum where larger materials are less degraded and smaller materials are more degraded. Smaller materials have amino acid compositions that suggest a preservation mechanism related to direct sorption to mineral surfaces. Larger materials may be preserved due to the presence of a hydrophobic moiety that results in the protein being excluded from the aqueous phase and protected from enzymatic attack in solution.

Sampling location

Sediments were collected off the coast of Washington aboard the R/V *Thomas G. Thompson* in July 2001. In this study we examined four shelf/slope stations ranging in depth from 120 to 1961 m (Table 1). The mineralogy and transport mechanisms for this location have been extensively studied (Nittrouer and Sternberg 1981; Sternberg 1986). Most of the sediment in this region is derived from the Columbia River (White 1970) and is transported to the north, parallel to the coastline, via strong bottom currents during storm events (Ridge and Carson 1987). Numerous organic analyses have also been performed on a variety of different sedimentary splits to examine the offshore biogeochemical preservation and degradation (Hedges and Keil 1995; Keil et al. 1998; Hedges et al. 1999).

Methods

Sediments were collected using the Model II-XT hydraulically dampened multicorer with polycarbonate core barrels. Only sediment cores with undisturbed sediment-water interfaces and a penetration depth greater than 30 cm were used. The oxygen penetration depths for the sediments were determined on each of the cores using a microelectrode (Hedges et al. 1999). Shortly after recovery, sediment cores were sliced into 5 cm intervals, taking care to remove any macrofauna, and placed in one or two 250 ml polycarbonate centrifuge tubes. The sediment was then centrifuged for 20 min ($16{,}500 \times g$) and pore water was removed. Samples were kept frozen prior to freeze-drying (72 h) at the University of Washington. Dried sediments were then lightly ground with mortar and pestle to homogenize the sample and break apart aggregates. Weight percent organic carbon and total nitrogen concentrations for these sediments were determined as described in Hedges and Stern (1984) and agree with previously published values from Washington coast (Table 1) (Hedges et al. 1999; Keil and Fogel 2001). Sediment accumulation rates for these stations were determined from a linear regression analysis of Washington coast rates versus distance offshore ($r^2 = 0.98$) (Hedges et al. 1999) and decrease offshore (Table 1). Oxygen exposure times for these sediments were calculated by dividing the oxygen penetration depth (cm) by the sediment accumulation rate (cm kyr^{-1}) (Hedges et al. 1999).

The proteinaceous component was isolated from the sediments using a non-hydrolytic base extraction (Nunn and Keil, submitted). Extractions were completed on approximately 50 mg of sediment from the 10–15 cm interval of all four stations. This interval was selected in order to assure that the sampled material was from beneath the mixed layer (as observed by a color change) (Nittrouer 1978) and had undergone prior diagenesis. We specifically sought to exclude freshly deposited material. Sediments were rinsed with 1 ml of nanopure water for 2 h on a shaker table (4 °C) to remove salts and the dissolved

Table 1. Details of station locations.

Station	Latitude N[a]	Longitude W[a]	Distance from shore (km)	Depth (m)	Porosity ϕ[b]	Surface Area ($m^2\ g^{-1}$)	%OC[c]	%TN[c]	THAA (mmol/gdwt)	DI[d]	OC/SA[e]	O_2 Penetration depth (cm)	accum rate (cm/kyr)[f]	OET[g] (year)
1	46.7775	124.5877	29.5	120	0.67	15.5	0.85	0.14	24.13	−0.61	0.97	0.48	14.2	33.3
2	46.7972	124.9066	53.8	434	0.62	17.2	0.90	0.14	21.97	−0.70	0.81	0.37	12.1	30.4
3	46.8002	125.2153	77.3	1029	0.88	29.5	0.82	0.10	12.47	−0.71	0.61	0.35	9.9	35.2
4	46.4260	125.5258	100.9	1961	0.83	33.0	2.25	0.27	31.33	−1.01	0.71	0.62	7.8	78.9

[a]In decimal degrees.
[b]Porosity determined on 10–15 cm interval.
[c]Weight percent OC and TN determined on 10–15 cm interval.
[d]Degradation Index (Dauwe et al. 1999); DI $>$ 0 indicates 'fresh' or labile OM, DI $<$ 0 indicates 'degraded' or refractory OM.
[e]Ratio of wt.% organic carbon to surface area.
[f]Accumulation rates from Hedges et al. (1999)
[g]OET (oxygen exposure time) calculated by dividing oxygen penetration depth by accumulation rate.

amino acid component. The slurries were centrifuged for 10 min (16,500 × *g*, 4 °C), the water was removed and put aside for amino acid analysis. The sediment was then treated with 0.5 ml freshly made 0.5 N NaOH for 2 h at 37 °C. Base extracts were isolated via centrifugation (10 min, 16,500 × *g*, 4 °C) and gradually diluted to a final concentration of sodium hydroxide that was acceptable to the Centricon centrifugal filtration devices used for size fractionations (< 0.14 N NaOH). To reduce aggregation and precipitation of proteins by rapid pH changes, the base-extract dilution was carried out by slowly adding 1 ml 0.1 N NaOH to the extract, gently stirring, followed by slow addition of 1 ml of nanopure water.

Size fractionation

Size fractionations of the base soluble sedimentary component were completed using Centricon centrifugal filter devices (Millipore). Three different molecular weight cut-offs were used, each with a specified centrifugation time and speed used for filtering (see below). Devices were first rinsed with 2 ml of 0.1 N NaOH followed by 2 ml of nanopure water, and stored wet. Sedimentary base extracts (2 ml) were transferred to the 2 ml sample reservoirs of the 100 kDa cut-off centrifugal filtration devices. Samples were centrifuged (1000 × *g*, 10 min, 4 °C) to reduce the volume and the remaining 0.5 ml of sample were added to the sample reservoir. Filtration with the 100 kDa unit was completed after an additional 30 min of centrifugation. Filtrate > 100 kDa was removed and combined with the filter. The filters were removed from the device with a pair of acetone-washed tweezers to reduce the loss of proteins adsorbed to the filter. Retentate was transferred to the 10 kDa cut-off sample reservoir and centrifuged (5000 × *g*, 1 h, 4 °C). Filtrate and filter were collected and retentate was transferred to the 3 kDa cut-off filtration device and centrifuged (7500 × *g*, 2 h, 4 °C). Once again, filtrate and filter and final retentate (< 3 kDa) were collected. All size fractions were frozen and dried under vacuum on a centrifugal-evaporator for ~8 h or until dry. Amino acids were analyzed on all fractions.

Amino Acid Analysis

Analyses of total hydrolyzable amino acids (THAA) were carried out on whole sediment samples from each station, size fractions, the remaining sediments after base extractions, and the initial water rinse prior to size fractionating. The method outlined by Cowie and Hedges (1992a) was used to quantify 3 non-protein amino acids and 15 common amino acids. Modifications to the method have been outlined in Nunn and Keil (submitted). Aspartic acid and glutamic acid mole percentages include contributions by the deaminated amino acids glutamine and asparginine (e.g. Asp + Asn = Asx), a typical artifact of the

hydrolysis method (Cowie and Hedges 1992a). While most of the literature for amino acids in marine sediments is subject to this same artifact (Keil et al. 2000), when necessary, comparisons to other data were made after literature amino acid distributions were artificially corrected (e.g. Asp + Asn = Asx).

To rapidly evaluate the quality, or character, of the amino acids in the different whole sediments and size fractions, the degradation index (DI) from Dauwe et al. (1999a) was employed. The index was developed from a principle component analysis (PCA) of the amino acid distributions of a variety of particulates, ranging from fresh phytoplankton to deep-sea sediments. A more positive signature is indicative of 'fresh' organic matter (e.g. phytoplankton), whereas negative signatures suggest the organic matter has undergone degradation and the components are less reactive or labile (e.g. deep sea sediments). Aufdenkampe et al. (2001) has shown that the DI index is not representative of diagenetic processes for riverine samples, and Ingalls et al. (2003) has cautioned against the over-interpretation of the index when comparing samples where the mineral matrix changes. Despite these caveats, there remains abundant evidence in the literature that a comparative diagenetic status can be determined from the index within a given environment such as the Washington margin (Keil et al. 2000; Van Mooy and Keil 2002; Van Mooy et al. 2002).

Results

Analyses of the 10–15 cm interval from each sediment core were used because we were interested in examining the precursors to long term preservation. This interval was chosen for several reasons; it is below the oxygen penetration depth ($\sim$1 cm) (Hedges et al. 1999), below the depth of severe mixing (biologically and physically mixed to depth of 5 cm) (Carpenter et al. 1982; Nittrouer et al. 1983), and is unlikely to be influenced by typical resuspension events (Nittrouer and Sternberg 1981). Several detailed analyses of Washington coast sediments have demonstrated that below the top few centimeters neither wt.%OC nor organic carbon to surface area ratios (OC:SA) change down-core, suggesting that minimal degradation or alteration of the organics takes place below the top few centimeters (Hedges et al. 1999). Surface area normalized carbon loadings are within the range commonly observed in continental margins (Mayer 1994; Hedges and Keil 1995), despite variations in bulk organic carbon content (0.82–2.24%OC) (Table 1).

Prior to base extraction, sediments were rinsed with water; this reversibly bound component contains 3–8% of the THAA. Base-extractions typically recover 25–35% of the THAA, and while the method is not completely effective, it is currently the most efficient method available for isolation of amino acids without hydrolysis (Nunn and Keil, submitted). The majority of the base-soluble proteins were in the two largest size fractions 10–100 kDa and $>$100 kDa (37–45% and 33–46% of base soluble fraction) (Figure 1). The distribution of amino acids in the total base-soluble component (sum of

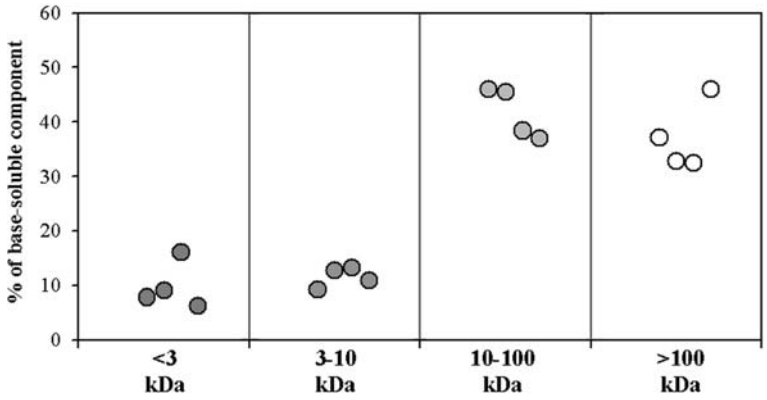

Figure 1. Proportioned recoveries of size fractionated base soluble proteins and peptides from stations 1–4 off the Washington coast. Percentages are relative to the total base soluble component recovered for each station. Data are shown for all 4 stations, beginning with the near-shore shelf station (left).

fractions) is comparable to that of the whole sediment THAA (Figure 2) (χ^2 test). The <3 kDa and >100 kDa fractions represent distinct components (χ^2 test; $p < 0.05$) (Figure 2 and 3). The 3–10 kDa size fraction may represent a smaller sized subset of the 10–100 kDa fraction, because these two fractions have similar amino acid compositions (χ^2 test).

The smallest size fraction is dominated by polar amino acids, making up ~45 mol%, and is enriched in non-protein amino acids (5.9–7.0 mol%) and basic amino acids (8.5–11.1 mol%) (Figure 2a, b, c, d, e). Glycine, a polar amino acid, is the dominant amino acid of the <3 kDa fraction, providing up to 30 mol% of the total amino acids (Figure 3b), whereas lysine is the dominant basic amino acid, enriched by 3–5 mol% in this fraction relative to the others. Acidic amino acids dominate the next largest size fraction, 3–10 kDa, consisting of 30–40 mol% aspartic acid (Figures 2c and 3c). Distributions of amino acids in the 10–100 kDa fraction also showed enrichments (16–28 mol%) in aspartic acid, with all other amino acids making up ≤ 10 mol% (Figure 3d). Relative to the other size fractions, the largest size fraction, >100 kDa, is enriched in non-polar amino acids (Figure 2); no particular amino acid statistically dominates the distribution (χ^2 test), but the combined mole percentages of the six non-polar amino acids make them the most prevalent.

DI values for the whole sediment THAA are very similar (-0.76 ± 0.17: SMD). The two largest size fractions contributing ~80% of the base-soluble component show offshore trends of decreasing DIs (Figure 2f). The degradation index in the 10–100 kDa size fraction is primarily controlled by the affect of the PCA coefficients (Dauwe et al. 1999a) for alanine and leucine. Analysis of the contributions of each of the amino acids in the >100 kDa fraction to the

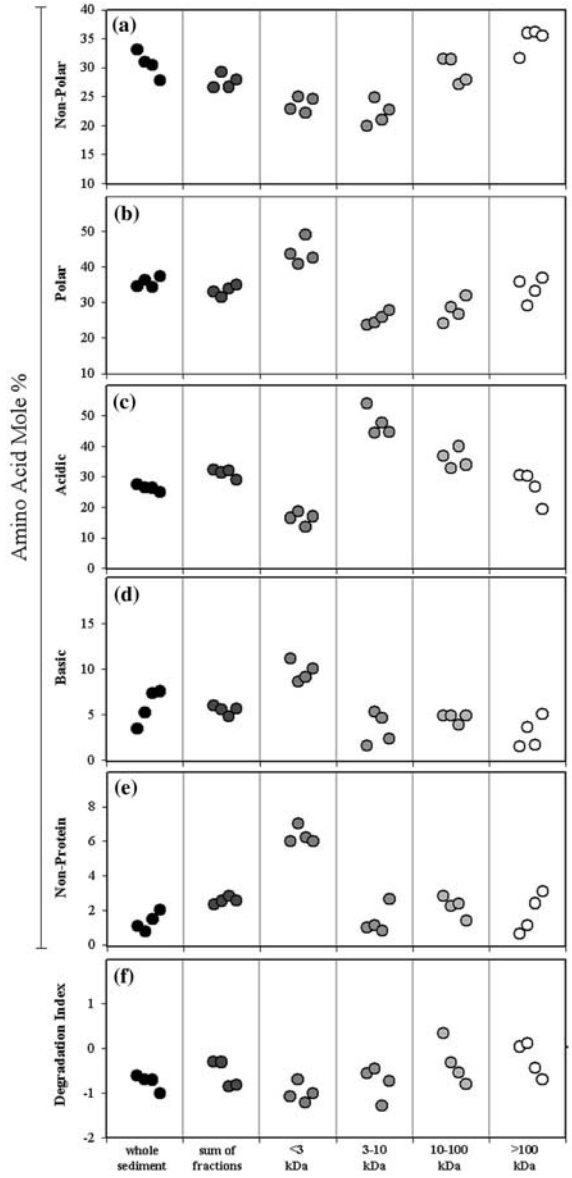

Figure 2. Relative distributions of amino acids based on the chemistry of their side chains. Mole percentages presented for whole sediment values (THAA), total base soluble fraction (sum of size fractions), and 4 different size fractions (< 3, 3–10, 10–100, > 100 kDa). (a) Non-polar (Ala, Ile, Leu, Met, Phe, Val). (b) Polar (Gly, Ser, Thre, Tyr). (c) Acidic (Glu, Asp). (d) Basic amino acids (Arg, His, Lys). and (e) Non-protein (α-Aba,γ-Aba, β-Ala). (f) Degradation index calculated according to Dauwe et al. (1999a). *Note scale differences*.

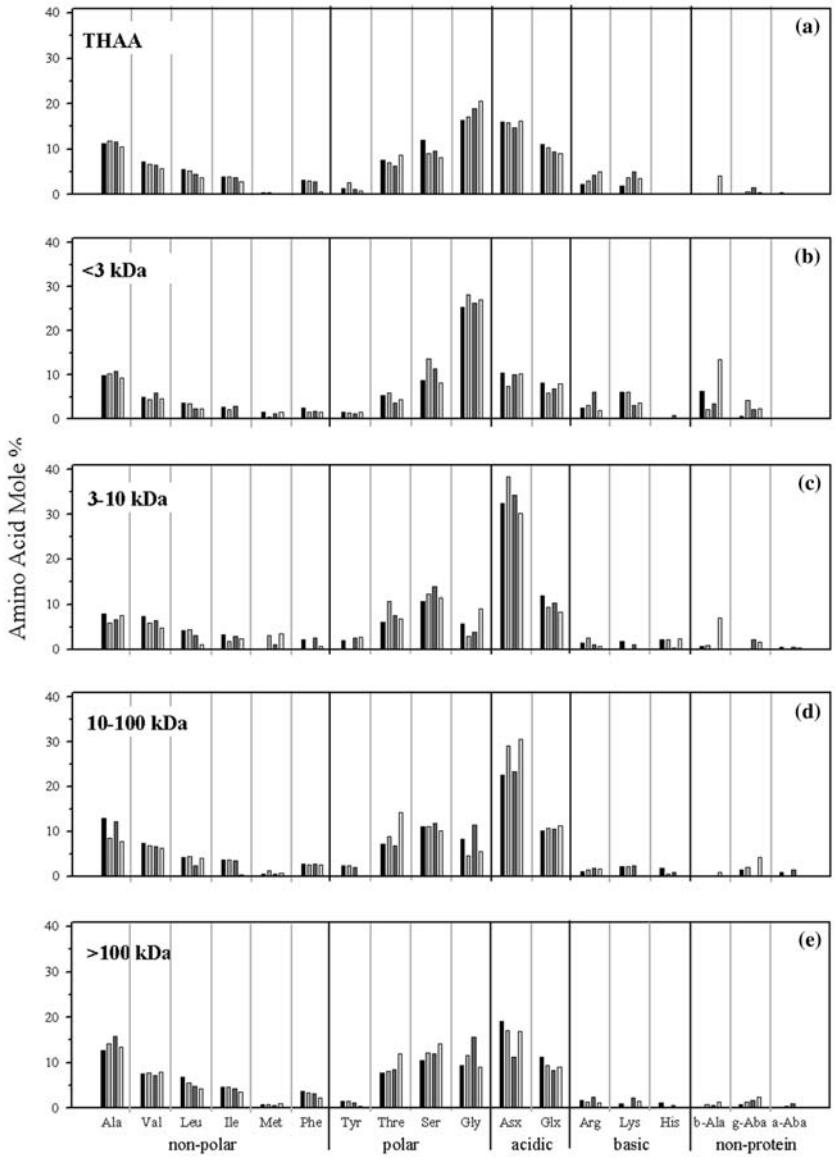

Figure 3. Histogram of mole percentages of the eighteen amino acids quantified for all four stations (stations 1–4 are represented from left to right by different colored bars). (a) Whole sediment hydrolysis (THAA). (b) < 3 kDa size fraction. (c) 3–10 kDa size fraction, and (e) 10–100 kDa size fraction. (f) > 100 kDa size fraction.

degradation index demonstrate that several amino acids are controlling the offshore behavior that lends to a more negative index (including, but not limited to Ala, Arg, Glx, Gly, Leu).

Discussion

Traditionally, amino acid geochemistry has been conducted using whole sediment acid hydrolysis where interpretations stress diagenetic change (Cowie and Hedges 1992b; Dauwe et al. 1999a). Typical diagenetic changes include enrichments in glycine and non-protein amino acids (Burdige and Martens 1988; Keil et al. 2000). By isolating different components and then separating by size, we are able to evaluate both diagenetic changes and the preservation of unaltered components (Nguyen and Harvey 1999). Previous estimates of the molecular weight distribution of extractable proteinaceous material indicate this component is predominantly >2 kDa in size (Nguyen and Harvey 1999; Pantoja and Lee 1999) and highly acidic (Carter and Mitterer 1978; Nguyen and Harvey 1999). Our current study is the first to show that the accumulation of amino acids indicative of diagenetic alterations occur in a distinct size fraction from the larger proteinaceous material that appears to be relatively unaltered.

< 3 kDa size fraction

On average, <3 kDa size fraction made up $\sim$10% of base-soluble amino acids, with a mean amino acid concentration of 0.49 mmol gdwt^{-1}. Glycine comprises up to 30% of the amino acids in this size fraction (Figure 3b), however glycine does not exist in concentrations $>25\%$ in any marine sources or other biological proteins (Cowie and Hedges 1992b). The enrichments commonly seen in sediments, in particular in the base-extractable fractions <3 kDa (Carter 1978), might result from the degradation of other amino acids. Glycine is the only amino acid created if the side-chain, or R-group, of any of the common amino acids is cleaved off. Keil and Fogel (2001) note the uncharacteristically variable δ^{13}C values of glycine in marine sediments relative to source materials. They proposed that this reflects production of glycine from other amino acids, which have an amino acid δ^{13}C range of greater than 14 per mil. To produce such variable δ^{13}C values for glycine, they hypothesized that glycine was highly reworked and 'synthesized' during diagenesis from other amino acids.

Several theories have been proposed, but it remains enigmatic as to why glycine is enriched in marine sediments. Dauwe and Middelburg (1998) have suggested that glycine escapes degradation because it is nutritionally outcompeted since it can be synthesized from other amino acids, however, Sugai and Henrichs (1992) demonstrated that glycine is reversibly bound to sediments and remineralized by bacteria at a comparable rate to other amino acids in pore waters. Instead, glycine not involved in peptide linkages might be enriched in our base-soluble <3 kDa fraction as a result of its ability to fit in tight places (Lodish et al. 2000; Zimmerman et al. 2004). Protection within mesopores would make it difficult for proteases to 'grab'

glycine, however it would be chemically extractable by base (Zimmerman et al. 2004).

The <3 kDa size fraction also has the highest concentration of non-protein amino acids (Figure 2e). Non-protein amino acids are typically absent in living organisms (Cowie and Hedges 1992b) and are indicative of extensive sub-oxic and anaerobic degradation and alteration of the different common amino acids or peptides (Lee and Cronin 1982). Similar to making glycine by removing the R-group, non-protein amino acids are produced via microbial decarboxylation reactions. In addition to being degradation products, non-protein amino acids are surface reactive (Aufdenkampe et al. 2001). Thus, it is logical that these common degradation products are enriched in the smallest size fraction since they are the products of hydrolysis and degradation.

Lysine, a basic amino acid, is also enriched in the <3 kDa fraction by 3.6–5.1 mol% relative to whole sediments and larger size fractions (Figure 3b). Although lysine is considered to be relatively hydrophobic and surface active as a free amino acid, lysine typically situates itself on the outside of large globular proteins (Janin 1979; Rose et al. 1985). Being on the outside of a globular protein would increase the chance for the residue to be cleaved from parent proteins as an individual amino acid or within a small peptide (Aguilar et al. 1998; Nunn et al. 2003). Proteases, such as Trypsin, cleave either before or after lysine, thereby increasing the probability of lysine-enriched peptides to occur in the smaller size fractions (see Nunn et al. 2003). The enrichment of lysine in the smallest size fraction increases the probability that these peptide components are strongly surface active. Nunn et al. (2003) incubated protein in marine sediments and observed the production of low molecular weight peptides. However, these low molecular weight molecules were not recovered from the pore water, suggesting that these fragments were either assimilated by bacteria or adsorbed to mineral surfaces. Peptides enriched in hydrophobic moieties are strongly surface reactive (Ding and Henrichs 2002); thus, the <3 kDa size can be characterized as containing reworked and surface active peptidic components.

3–10 kDa & 10–100 kDa size fractions

The two intermediate size classes do not have significantly different distributions of amino acids (χ^2 test; $p = 0.05$). The 3–10 kDa fraction, however, is a notably smaller fraction of the total base-soluble pool, contributing only $\sim$10%, whereas the 10–100 kDa fraction contributes $\sim$42%. Both of these fractions are highly acidic, with aspartic acid contributing 38 mol% of the amino acids (Figure 3c, d). Comparisons of several hundred sequenced biological proteins demonstrate that aspartic acid is frequently the dominant amino acid (http://us.expasy.org/sprot). There are several sedimentary and marine water column investigations with evidence that also corroborate these findings (Carter and Mitterer 1978; Henrichs and Farrington 1987; Dauwe et al. 1999b; Ingalls et al. 2003). Alternatively, the 3–10 kDa size class may be

comprised of degraded products of the 10–100 kDa size class. Since aspartic acid is situated within the hydrophobic cores of globular proteins (Janin 1979; Rose et al. 1985) and these proteins are degraded from the exterior inward (Aguilar et al. 1998; Nunn et al. 2003), a decrease in molecular weight should correspond to an enrichment in aspartic acid.

> 100 kDa size fraction

The enrichments observed in the smaller size fractions were not observed in the largest size fraction >100 kDa (Figure 3e); no one particular amino acid makes up >20% of the amino acid distribution. Nguyen and Harvey (2001) have previously suggested that larger size fractions are aggregates of smaller sized fractions. We attempted to model the amino acid composition of the large size fraction by mixing different percentages of the three smaller size fractions, but the amino acid distribution of the >100 kDa size fraction cannot be achieved in this manner. Thus, the >100 kDa fraction does not result from a simple mixture of the smaller peptides and proteins extracted from Washington coast sediments. The composition of the >100 kDa fraction, and in fact all fractions >3 kDa, suggest the preservation of intact proteins (see discussion below).

Chemistry of base extractable proteins

Although it is difficult to discern how many different types of proteins or peptides are present in the fractions, we can gain a better understanding of the chemistry of each isolate by assuming that each fraction represents a single type of protein and then employing a set of biochemical approaches. The amino acid composition of a protein can control its three dimensional structure and its physical location within a cell (Voet and Voet 1990). For example, large contributions by hydrophobic amino acids (non-polar) can cause the amino acid chain to fold inward on itself. Therefore, assessment of the ratio of non-polar to polar amino acids can provide information on the tertiary structure of the protein (Voet and Voet 1990). Ratios >1, such as those calculated for the >10 kDa (1.06) and >100 kDa (1.03) fraction, are indicative of proteins with globular structures. The larger the protein, the smaller the ratio can get and still be representative of a globular protein (Voet and Voet 1990). However, for the two smallest size fractions this estimation yielded lower values, indicating globular structures are less likely for these peptide-sized fragments (<3 kDa: 0.5; 3–10 kDa: 0.8).

Another parameterization of the chemistry of the sedimentary proteins can be made by comparing the number of acidic to basic amino acids; this ratio can provide information on the isoelectric point (pI) of the proteins in each fraction to help decipher how to chromatographically isolate the different fractions

(Kaufmann 1997; Schaller et al. 1997; Whitelegge et al. 1998) and to get information on cellular location (Schwartz et al. 2001). In the three largest size fractions, this ratio is >2, indicative of proteins with an acidic pI. If we also assume that different size classes represent one protein or a small suite of similar proteins, an estimation of the isoelectric point (pI) for the proteinaceous component of the different size classes can be calculated from the mole percent distributions of all the amino acids (Bjellqvist et al. 1993; Wilkins et al. 1998). For this estimation, each size class of proteins was assumed to have 1000 amino acids (allowing amino acid mol% fractions to be accounted for). Amplifying the number of amino acids to match the appropriate molecular weight for each size class does not alter the calculated isoelectric points because this type of estimate examines the net charge of the protein and does not take into account protein folding on ionizable groups. Sillero and Ribeiro (1998) demonstrated that these theoretical calculations of isoelectric points are close to experimentally determined values. All four isoelectric points calculated for the different size fractions were acidic (Table 2). Although it is highly unlikely the <3 kDa fraction represents one protein, it yielded the highest pI (4.4) (Table 2). The larger size fractions generated very similar pIs (in order of increasing size: 3.01, 3.32, 3.27). Nguyen and Harvey (1999) isolated several proteins from an algae-dominated lake; most of the sedimentary proteins reported from Mangrove Lake sediments had molecular weights from <6.5–37 kDa and pIs of 3.8 to 5. The 2D gel electrophoresis method they employed also recorded an acidic front with no discernable proteins. Although all the proteins they observed were also acidic, our calculated isoelectric points for the proteins in Washington sediments are lower; therefore we examined these theoretical pI values to determine if they were low as a result of the overestimation of acidic residues from the hydrolysis method of analysis (mentioned above). By looking at the percent occurrence of each individual amino acid in nature, the acidic residues, Asp and Glu, are more prevalent than Asn and Gln (Beavis and Fenyo 2003). Therefore, as a rough estimation to attempt to correct for the acidic residue overestimation inherent to hydrolysis, we recalculated the pIs assuming 50% of the aspartic acid was asparginine (Asn pI 5.52), and 50% of glutamic acid was glutamine (Gln pI 5.52). The resulting new pIs for each size fraction were 9.3, 3.35, 3.69, and 3.64 (in order of increasing size). Even with 50% reductions in aspartic acid in the three larger fractions, over 15 mol% of the amino acids remain acidic (pI $\sim$ 3). The smallest size fraction, <3 kDa, has the most dramatic change in calculated pI because glycine (pI $\sim$ 5) is the dominant amino acid ($>25\%$), lysine (pI $\sim$ 10) makes up 5% of the amino acids, and it is not dominated by acidic residues. Even if we reduce the number of acidic amino acids by 75%, the pI of the larger size fractions do not change, likely a result of the low mole percent of basic residues.

Several studies have been carried out to determine the relationship of a proteins' pI to its cellular localization (Van Bogelen et al. 1999; Schwartz et al. 2001) for bacteria, archae, and eukaryotic proteomes. Although there are slight

Table 2. Chi squared comparison of amino acid distributions of base soluble size fractions to known individual proteins, diatom cultures, and oceanic samples.

ID	MW[a]	pI[b]	Size fractions				Description (index)[c]
			pI 3.27	pI 3.32	pI 3.01	pI 4.4	
			>100 kDa	10–100 kDa	3–10 kDa	<3 kDa	
Bacteria							
Invasion Protein	116.2	5.33	75.6%	74.7%	48.2%	70.5%	Outer-membrane protein exposed to the bacterial surface (Q99Q93)
Fe-transporter	81.2	4.75	**80.5%**	**82.1%**	49.5%	41.6%	Outer-membrane Fe-receptor (P16869)
Bact.Average			**79.0%**	65.2%	10.8%	52.7%	Average value for three bacterial cultures
Bacterial porns							
Porin P.A1	45.8	7.11	73.5%	64.6%	20.4%	64.9%	*Pseudomona aeruginosa* Porin (Q9HUR5)
Porin P.A2	47.3	6.81	65.8%	52.2%	<10%	60.1%	Pseudomona aeruginosa Porin (Q910E2)
OMP *C E. coli*	40.3	4.51	72.5%	75.2%	46.1%	59.9%	*E. coli* Porin C precursor (Q9K597)
Porin V.P.	29.9	4.34	**83.6%**	**81.0%**	48.0%	66.8%	*v. parahaemolyticus* Porin S protein (Q56714)
Porin Omp.E.C.	40.5	4.55	73.9%	75.7%	45.4%	60.4%	*E. coli* Porin Omp C (Q8XE41)
Porin Omp.F	39.3	4.76	73.3%	71.4%	34.1%	62.8%	*E. coli* Porin Omp F (P02931)
Porin Average			**78.9%**	76.3%	41.0%	65.8%	Average porin (above $n = 6$)
Diatom proteins							
Silaffin	27.5	10.02	9.2%	22.4%	<10%	40.6%	Silaffin protein *C. fusiformis* (Q9SE35)
Si-transporter	60.6	5.48	61.5%	42.1%	<10%	21.2%	Silica transporter-*C. fusiformis* (O81199)

Diatom Frustlin	45.9	4.37	**80.1%**	**82.1%**	59.1%	69.1%	Alpha 2 frustulin protein *C. fusiformis* (Q39495)
Ca-binder	46.2	4.26	**81.7%**	**83.5%**	61.1%	70.6%	Ca-binding glycoprotein *C. fusiformis* (Q39494)
Hep 200	101.0	4.00	**77.9%**	**81.7%**	56.6%	14.5%	Intracellular component *C. fusiformis* (O22015)
Hep B	95.3	3.99	64.5%	76.1%	63.2%	10.4%	HEP B associated with silica cell wall *C. fusiformis* (O22016)
Hep C	83.7	3.91	**79.4%**	**80.8%**	54.6%	17.4%	HEP C assoc. w/ silica cell wall - *C. fusiformis* (O22017)
Bulk diatoms							
C. gacilis[d]			**88.1%**	**81.8%**	42.4%	64.3%	Hydrolysis of *C. gacilis* culture
C. gradiatus[d]			**88.6%**	**77.3%**	29.1%	55.0%	Hydrolysis of *C. gradiatuss* culture
T. pseudonana[d]			**92.1%**	**77.6%**	32.1%	75.9%	Hydrolysis of *T. pseudonana*culture
Average Diatom			**91.2%**	**80.4%**	35.9%	63.8%	Average of diatoms (above $n = 3$)
Phytoplankton[e]			**86.3%**	73.0%	21.3%	55.3%	Average for net and trap samples of phytoplankton
Zooplankton[f]			**81.8%**	67.1%	15.0%	68.9%	Hydrolysis of *Calanus pacificus* culture

[a]Molecular weight of individual proteins reported in $\times 10^3$.
[b]Isoelectric point of individual proteins (from SWISS-PROT database).
[c]ID number for individual proteins; corresponds to SWISS-PROT database (http://us.expasy.org/sprot/).
[d]Data from complete hydrolysis of culture (Ingalls et al. 2003).
[e]Data from complete hydrolysis sediment trap samples (Cowie & Hedges 1992b).
[f]Data from complete hydrolysis of zooplankton samples (Dauwe & Middelburg 1998).

variations between different domains of life, in general the isoelectric points for these proteomes center either around pI 5 or 9 (Schwartz et al. 2001). Schwartz et al. (2001) concluded that this bimodality is required for different protein functions depending on its subcellular localization; an isoelectric point around pI 5 is indicative of cytoplasmic proteins, while pI 9 is typical of integral membrane proteins. Extrapolating this idea to our data leads to the hypothesis that the protein component we are extracting from the 3 largest size fractions are likely located in the cytoplasm rather than the membrane. Previous marine research has demonstrated that there is preferential preservation of bacterial membrane components in the water column (Tanoue 1996; McCarthy et al. 1998). The base-soluble proteinaceous fraction from Washington coast sediments, however presents evidence that preservation of proteins or peptide fragments from phytoplankton cytoplasm may also contribute a significant fraction to the total sedimentary carbon pool (Arnarson and Keil submitted; Nguyen and Harvey 1997; Satterberg et al. 2003). Since higher isoelectric points are indicative of membrane components (Schwartz et al. 2001), it is possible that the bacterial membrane proteins will not solubilize in base; this would suggest that membrane components may be present in the remaining THAA that was not extractable by base. Both of the studies that look at protein preservation during phytoplankton decay using 2D gel electrophoresis also revealed preservation of acidic proteins (Nguyen and Harvey 1999; Robbins and Brew 1990). Gaining chemical insights, such as relative size and charge, will help us during the separation and isolation of the individual proteins preserved in sediments. Chemical similarity of the four offshore Washington coast stations and the distinct differences between the four size classes will simplify eventual isolation and analyses of sedimentary proteins.

The distinct distributions of the amino acids for each size fraction are suggestive of preservation of a particular protein or suite of proteins. To test this hypothesis we compared our amino acid distributions (mol%) to sequenced proteins from bacterial membranes, cultures, and porins, intra cellular and silica-bound diatom proteins, bulk phytoplankton, and bulk zooplankton (Table 2). Individual proteins evaluated range in size between 27 and 116 kDa and pI 3.9 and 10.0. A chi squared (χ^2) test was carried out on the mole percent data to determine the significant differences between our observed distributions and expected amino acid distributions if we assume the protein was one of the 23 we examined from the literature. The scores were calculated by subtracting the chi squared value from 100%. Therefore, an exact match would have a score of 100%. Proteins are not statistically different if the score is $>76.5\%$ ($p = 0.05$; $df = 14$) (Table 2). None of the proteins were statistically similar to amino acid compositions found in the <3 kDa and 3–10 kDa size fractions (Table 2). In general, the diatom proteins, both bulk values and individual proteins, had the most similar amino acid compositions and isoelectric points compared to the two largest size fractions of sedimentary proteins (10–100 kDa and >100 kDa; Table 2). There were, however, representative proteins for the different sub-groups that were statistically similar. For example,

the >100 kDa fraction demonstrated statistical similarities with average bulk values for all six sub-groups of proteins (i.e. Bact. Average, Porin Average, Diatom Average, Phytoplankton and Zooplankton; Table 2). Several studies have proposed bacterial membranes dominate the preserved protein fraction in waters (McCarthy 1998; Tanoue 1996), whereas the base-soluble fraction analyzed in this study demonstrate that the sedimentary proteins preserved are also statistically (χ^2 test) similar to phytoplankton or diatom amino acid compositions (Table 2). A graphical comparison of the amino acid mole percentages of the two largest size fractions to the two proteins with the highest score illustrates how the distributions are comparable (Figure 4). It is unlikely that the *T. Pseudonana* cultures, the most statistically similar amino acid

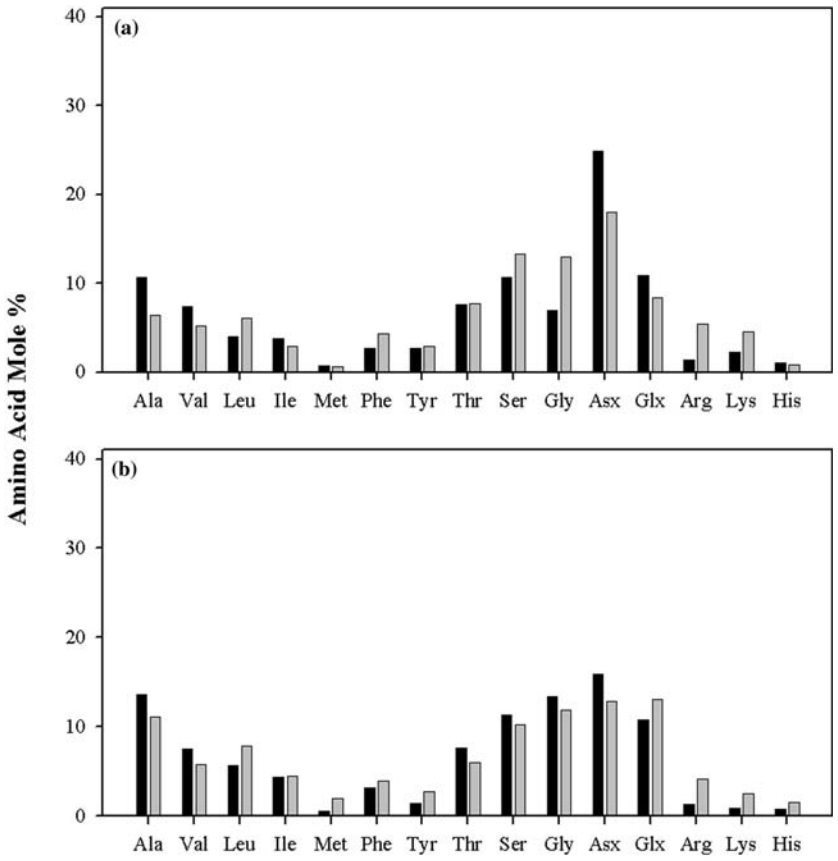

Figure 4. Comparison of the 10–100 kDa and >100 kDa size fractions (amino acid mole percentages) (black bars) to source proteins with the best score (grey bars) (see Table 2). Amino acid distribution of (a) 10–100 kDa protein fraction vs. the Ca-binding glycoprotein from the marine diatom *C. fusiformis* and (b) >100 kDa protein fraction *vs*. average composition of the marine diatom *T. pseudonana*.

distribution to the >100 kDa fraction, is made up of proteins that are all >100 kDa. However, we are unable to decipher if the aggregation of proteins other than those we see in the smaller size fractions might aggregate to yield a >100 kDa fraction. As mentioned earlier, Nguyen and Harvey (1999) have suggested that some of the larger protein molecular weight fractions observed in their studies might represent protein–protein aggregates or cross-linked peptides with or without carbohydrates (Yamada and Tanoue 2003). The main point is that these larger fractions are not composite aggregates of the smaller degraded material (e.g. <3 kDa) present in these sediments. Instead, the analyses of amino acid distributions of the two large size classes of sedimentary proteinaceous material can be accounted for by assuming these fractions consist of viable source proteins, without involving a diagenetic explanation.

Preservation mechanisms

Approximately 45% of all organic carbon preserved in the oceans accumulates and is eventually preserved along continental margins (Berner 1982). Although this is the dominant reservoir of available carbon, the processes that dictate the ultimate fate of this material remain contentious. There are five prevailing mechanisms proposed to promote preservation of organic matter in marine sediments: entrapment in organic matrix (Knicker and Hatcher 1997; Zang et al. 2000; Zang et al. 2001), entrapment in biominerals (Sykes et al. 1995; Chen et al. 2000; Ingalls et al. 2003), biomolecule aggregation (Benner et al. 1992; Chin et al. 1998; Nguyen and Harvey 2001), intrinsically refractory structures (Henrichs 1992; Tanoue et al. 1995; McCarthy 1998), and sorption to particles (Arnarson and Keil submitted; Henrichs and Sugai 1993; Keil et al. 1994). In the following discussion, we try to determine the mechanisms responsible for the preservation of the base-soluble proteins in Washington coast sediments. We are able to eliminate three of these mechanisms for the base-soluble protein fraction and propose how the other two might work together, playing an integral role in the long-term preservation of these sedimentary proteins.

The inability to analytically characterize all the nitrogen (TN) present in sediments initiated a series of investigations that looked at the preservation potential of peptides and proteins trapped inside of organic matrices (Knicker 2000; Zang et al. 2000; Hedges et al. 2001a). Nunn and Keil (submitted) attempted to extract the majority of the sedimentary nitrogen component for amino acid analysis by examining the efficiency of six different solvents along with their combined effects in sequential extractions. They demonstrated that the base-soluble component was most similar in amino acid distribution to the whole sediment THAA, however they state that the base-extraction is a 'soft' technique, accounting for ~10% of the TN, and it is probably unable to account for any amino acids trapped in organic matrices. Thus far, the organic-trapped nitrogen component remains to be successfully extracted for amino

acid analyses or quantified (Knicker and Hatcher 1997; Nunn and Keil submitted). Therefore, there might be organic material entrapped in these sediments, but this preservation mechanism can not account for the base-soluble proteins observed in this study.

Proteins trapped within natural mineral matrices, such as frustrules, also provide a means for preservation. Biominerals have been noted to act as excellent ballasts (Hedges et al. 2001b), shuttling organic matter to depth in a matter of days, while protecting potentially labile material from degradation (Ingalls et al. 2003). However, for this study, we did not employ a protocol to release a large fraction of the proteins bound or trapped within silica or carbonate (King 1974; Nunn and Keil submitted).

Aggregation of biomolecules has been observed in many systems Williams and Keil 1997; Chin et al. 1998; Nguyen and Harvey 2001). Organic cross-linkings and condensation reactions could make previously labile macromolecules unrecognizable or undegradable by enzymes (Nguyen and Harvey 2001; Zang et al. 2001) and ultimately biologically unavailable. This mechanism has been used to explain the recurring observation that an increasing percentage of the residual nitrogen from degradation experiments and present at depth in sediments is in the largest size fraction (>100 kDa) (Pantoja and Lee 1999; Nguyen and Harvey 2001). Aggregation of small biomolecules would also increase their surface affinity and potential for sorptive preservation (Collins et al. 1992; Schuster et al. 1998). However, the mixing model mentioned previously was conducted to test this mechanism as a potential method of creating the largest size fraction observed in the Washington coast sediments. Each size class has a unique and distinct signature and can not be modeled through a simple mixing of the different classes. Biomolecule aggregation of the small base-extractable components, therefore, does not represent the majority of larger protein components present in the base-extractable fraction of margin sediments. However, the >100 kDa size fraction likely includes some aggregated material since there are relatively few proteins of this size that occur in nature (Lodish et al. 2000). With the current method of chemical and data analysis we are unable to completely eliminate aggregation of other source proteins as a possible mechanism.

Two of the originally proposed mechanisms remain as viable means to explain the preservation of the base extractable proteins along the Washington coast: intrinsic stability of compounds and sorptive preservation of compounds. Based on previous studies, it is unlikely that a significant fraction of the proteins and peptides from natural marine sources are *intrinsically* stable (Hollibaugh and Azam 1983; Nguyen and Harvey 1997; Pantoja 1997). Investigations have demonstrated that they can be aggregated or modified, yielding refractory compounds (Keil and Kirchman 1994; Nguyen and Harvey 2001), but naturally resistant proteins not bound within a matrix or polymerized have yet to be discovered. An alternate explanation might include the *intrinsic* preference for a protein or peptide to adsorb to a particle surface because this can reduce its chance to be degraded (Keil et al. 1994; Pantoja

1997). Keil et al. (1994) demonstrated that the adsorption of organics to mineral surfaces can reduce the remineralization rate by five orders of magnitude. There are also several recent studies that have demonstrated the potential for various compounds to preferentially partition into the solid phase from the dissolved phase of a system (Arnarson and Keil 2000; Ding and Henrichs 2002; Satterberg et al. 2003). For example, the majority of phytoplankton exudates (47–85%) and peptides are surface reactive with partition coefficients that greatly exceed those found for natural pore water DOC (Ding and Henrichs 2002; Satterberg et al. 2003). Enrichments of organic matter on mineral surfaces can also catalyze particle aggregation, further protecting the organics (Arnarson and Keil submitted; Hamm 2002).

Synthesizing our findings with previous research, we have developed a scenario that can explain preservation of base-extractable amino acids in marine sediments. We propose that planktonic cytoplasmic proteins and peptides are rapidly sorbed to particles (Satterberg et al. 2003) and shuttled through the water column to the sediment surface. A large fraction of these proteins are either completely or partially adsorbed to minerals. Hydrophilic domains of some of the larger adsorbed proteins are exposed to the solvent, making cleavage points easily accessible. These hydrophilic domains are removed from the parent protein and some are released as small peptides (< 3 kDa) into the pore water, where their component amino acids can be stripped of their R-groups or decarboxilated, yielding peptide fragments enriched in glycine and non-protein amino acids. This proposed scenario suggests that the adsorption of labile proteins to mineral grains might be the dominant mechanism controlling preservation of base-extractable THAA.

We further suggest that the largest protein components resemble the composition of unaltered, undegraded proteins while the smaller fragments are degradation byproducts and surface active peptides. This agrees well with Amon and Benner's (1996) conceptual size continuum model for dissolved organic matter, in which larger material reflect unaltered sources and smaller materials reflect highly reworked degradation products. Given that there are 150×10^{15} grams of organic carbon present in the top 1 m of ocean margin sediments (Hedges and Keil 1995), and assuming 15% of this carbon is amino acid-carbon (Keil et al. 2000), then the amount of base-extractable protein > 10 kDa in the top 1 m of ocean margin sediments can be estimated to be 5×10^{15} g C. This estimate is equal to, or greater than, the total amount of protein in either marine biota or terrestrial biomass (1×10^{15} and 3×10^{15} g C, respectively) (Li 2000), thus emphasizing the need to better understand this large pool of marine detritus.

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