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A comparison of non-hydrolytic methods for extracting amino acids and proteins from coastal marine sediments

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Abstract

Advances in analytical techniques now allow for the potential analysis of intact peptides and proteins isolated from marine sediments. However, there is no established technique for the extraction of macromolecular materials from marine sediments. Six different methods for extracting the amino acid component from coastal marine sediments were compared to the standard hot acid hydrolysis technique for their percent recovery and amino acid composition. The standard hot acid hydrolysis on dried, whole sediments released the greatest concentration of total amino acids (*PS*-THAA; 3.52 mg $gdwt^{-1} \pm 10\%$ (SMD)), yet this only accounted for 22% of the total nitrogen in Puget Sound sediments (Washington, USA). Repeated hydrolysis of the same samples did not improve the recovery of nitrogen by more than an additional 10%. Base extraction (0.5 N NaOH) was the second best method for recovering amino acid nitrogen, releasing 60% of the Puget Sound total hydrolyzable amino acids (*PS*-THAA) (corresponding to 13% of the total sedimentary nitrogen), and has the advantage that it does not rely on peptide hydrolysis to free the nitrogenous component from the sediment matrix. The amino acid distribution of the 0.5 N NaOH extract was not significantly different than the initial THAA. Other non-hydrolyzing methods released lower yields of amino acids (Triton X-100 ≥ hot water > 50 mM NH_4HCO_3 > HF), but might prove to be of use to investigators interested in specific fractions of sedimentary organic nitrogen because these four methods had distinctly different amino acid compositions (enrichments in basic amino acids and depletions in acidic amino acids). Treatments with HF both before and after traditional hydrolysis and/or extractions with base did not release any more of the sedimentary nitrogen. Our results are consistent with the hypothesis that a large fraction of the sedimentary nitrogen (TN) is protected within an organic matrix.

Keywords: Protein; Amino acids; Marine sediments; Biogeochemistry; Mass spectrometry

1. Introduction

Most nitrogen in marine sediments is thought to be in amide form (Knicker, 2000; Zang et al., 2001), and most nitrogen in planktonic sources is as amide-linked protein (Sharp, 1983), leading to the hypothesis that much of the organic nitrogen in marine sediments is proteinaceous (Pantoja and Lee, 1999). Although there have been several techniques used to extract intact proteins from sediments (Ding and Henrichs, 2002; Gelinas et al., 2001; Henrichs and Farrington, 1987; Ingalls et al., 2003; Kröger et al., 1997), to date no one has examined how well these techniques work in mineral laden sedimentary systems. In fact, most previous examinations of the sedimentary nitrogen component have relied upon acid hydrolysis. The traditional acid hydrolysis technique, which destroys most amide bonds, yields total hydrolyzable amino acid concentrations (THAA) that typically represent only ~40% of the

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sedimentary nitrogen (Keil et al., 2000). Thus, the most efficient approach for extracting organic nitrogen from sediments yields less than half the parent material and destroys information about source. This renders acid hydrolysis unacceptable as an isolation tool prior to evaluation of sedimentary organic nitrogen using advanced macromolecular analytical techniques such as gel electrophoresis or mass spectroscopy (Nunn et al., 2003; Ostrom et al., 2000).

Alkali extractions are the most commonly employed methods for solubilizing organic matter by sediment geochemists (Henrichs and Sugai, 1993; Mayer et al., 1986; Pantoja and Lee, 1999), however several other methods have also been explored. Nguyen and Harvey (1998) modified a one-step alkali extraction by first precipitating the proteins and rinsing away smaller, soluble organic matter. Heating sediments in distilled water and dilute detergents can also be used to solubilize particulate proteins (Ding and Henrichs, 2002; Henrichs and Farrington, 1987; Keil and Fogel, 2001) and prevent them from aggregation or adsorption to tubes, membranes, or other surfaces (Gharahdaghi et al., 1994; Rosinke et al., 1995). A distinctly different approach is to use hydrofluoric acid (HF) to isolate organic matter by dissolving the mineral matrix (Bremner and Harada, 1959; Gelinas et al., 2001). This method also has the potential to release specific proteins bound within or trapped in silica or opaline cell walls (e.g., Ingalls et al., 2003; King, 1974; Kroger et al., 1997). Of the methods examined, 0.5 N NaOH was the most efficient method for releasing amino acids, and it released a THAA with an amino acid signature very similar to whole sediment acid hydrolysis.

In this manuscript we evaluate six extraction techniques designed to remove nitrogen based organic matter from sediments, and we compared resultant nitrogen and amino acid yields to the standard acid hydrolysis method. Because we are interested in the eventual analysis of unhydrolyzed sedimentary proteins, we only examine 'soft' extraction methods that maintain the integrity of the macromolecules. The methods we test include procedures frequently used for determining surfactant-soluble proteins (Cumming and Iceton, 2001), base-soluble proteins (Henrichs and Sugai, 1993), proteins >2 kDa (Nguyen and Harvey, 1998), and silica-bound (or trapped) proteins (Gelinas et al., 2001; Kröger et al., 1997). The goals of the research are to determine which technique extracts the greatest component of the hydrolyzable amino acid pool, and to determine if different extraction techniques isolate different components from sediments. We used amino acid analyses of acid hydrolyzed extracts to determine whether there was a difference in the amino acid composition extracted by various methods.

2. Methods

Sediment was collected in Puget Sound (Washington) due west of Carkeek Park, Seattle (47 43N 122 24.5W) from a depth of 98 m aboard the University of Washington's R/V Barnes. A Soutar box core was subsampled with an acrylic core tube (ID=8 cm). Overlying water was removed and the first 12 cm of the sub-core were sieved (mesh size: 600 μm) to remove any macro and meio-fauna. The overlying water was used to help wash the sediments through the sieve. Once sieved, the sediment was transferred to 250-ml polycarbonate tubes and centrifuged (10,000×g; 20 min; 4 °C) to isolate the solid phase. The sample was then freeze-dried for 72 h and lightly ground with a mortar and pestle to homogenize the sample and break apart aggregates.

Average weight percent organic carbon and total nitrogen were determined in triplicate for the homogenized Puget Sound sediment (~20 mg). Samples were acidified to remove inorganic carbon and analyses were made at the University of Washington following the method outlined in Ward et al. (2001).

2.1. Sediment extractions

Six different extraction methods were tested for isolating amino acids in tact from sediments (0.5 N NaOH, 0.1 N NaOH, TritonX-100, hot water, NH_4HCO_3, HF). The methods we examined were chosen based on frequency and familiarity within the marine and biochemistry communities, reduction of potential contaminants for eventual analysis using mass spectrometry, and ease of use (Cumming and Iceton, 2001; Gelinas et al., 2001; Nguyen and Harvey, 1998). Fewer steps during the extraction procedures were favored to reduce the potential loss of sample. Each method was conducted, at least in duplicate, on ~20 mg of freeze-dried Puget Sound coastal sediment. For all slurries, the soluble fraction was isolated from the remaining particulates by centrifugation at 10,000×g for 15 min (4 °C). All fractions were then frozen and dried under vacuum on a roto-evaporator for ~8 h, or until dry.

For base extractions, 2 ml of either 0.5 N NaOH or 0.1 N NaOH (pH>11) were added to the sediments. Slurries were then vortex mixed and sonicated in a water bath at 37 °C for 2 h. Secondly, similar extractions with Triton X-100 (0.2%, Sigma Aldrich) and hot

water (nanopure) extractions were conducted: 2 ml of the solvent were added to the sediments and shaken while heated at 95 °C for 1 h.

We also examined the effectiveness of the method used by Nguyen and Harvey (1998) to extract the larger proteinaceous component (>2 kDa) in Puget Sound sediments. First, small peptides and non-proteinaceous organic matter were removed by intermittently shaking samples with 2 ml of acetone (0.1% β-mercaptoethanol containing 10% (w/v) trichloroacetic acid, TCA) for 1 h at −20 °C. TCA promoted precipitation of the larger protein components, so they were not solubilized. The slurries were then centrifuged and the supernatant was removed. This 'rinsing' step was repeated twice more, once with and once without TCA. All 'rinses' were pooled to determine the fraction of amino acids removed in this step. The remaining solids were then extracted using relatively mild conditions (compared to a traditional base extraction). Two ml of 50 mM NH_4HCO_3 (pH=7.8) was added to the 'acetone-rinsed' sediment. The supernatant was collected after sonicating in a water bath at 37 °C for 2 h. Although all fractions were analyzed for THAA, the second solubilization with NH_4HCO_3 is thought to contain the extractable, intact larger protein (Nguyen and Harvey, 1998).

Sediments were also extracted with HF using a modification of the demineralization protocol of Gelinas et al. (2001) to remove any silica-bound proteins (Kröger et al., 1997). Briefly, sediments were mixed with 2 ml of 1 N HCl and 10% (v/v) HF solution and placed for 12 h on a shaker table at 20 °C. The slurry was then centrifuged at 1000×*g* for 15 min (20 °C) and the HF soluble fraction removed. The 12 h extractions were repeated with 1.5 ml and 1 ml of the HF solution on the remaining residue, and all HF soluble fractions were pooled to give a single sample.

Lastly, we tested combinations of the above extractions to determine if sequential extractions were more effective at releasing amino acids from sediments. Two combinations tested whether treatment with either HF or NaOH released additional amino acids after the initial hydrolysis. Sediments were first hydrolyzed with HCl (HYD). The hydrolysate was removed by centrifugation, and the particulate matter was neutralized and treated with either NaOH (BASE) or hydrofluoric acid (HF) as described above. The soluble portion was then isolated via centrifugation and the remaining particulate matter was hydrolyzed again (sequences; HYD–BASE–HYD or HYD–HF–HYD). All fractions were analyzed for amino acids. Another combination determined whether combining NaOH and HF treatments assisted in the liberation of amino acids. Sediments were first treated with HF, and the soluble fraction was removed by centrifugation. The particulate matter was then subjected to an extraction with NaOH, and the soluble portion was separated from the particulate fraction by centrifugation. The remaining particulate fraction was hydrolyzed (HF–BASE–HYD). The final combination-experiment followed the same concept as the previous experiment, however the order of HF and NaOH treatments was reversed (BASE–HF–HYD). Replicate experiments were also conducted with this combination, however one replicate was lost.

2.2. Amino acid analyses

Although there are several assays and other molecular methods for determining protein concentrations (e.g., BCA, CBQCA, Coomassie blue, gel electrophoresis), these methods are dependent on the size and charge relative to the standards they are compared to and do not tolerate natural organic contaminants that are frequently present in marine sediments (Mayer et al., 1986). To consistently determine the effectiveness of the different protein extraction techniques, total hydrolyzable amino acids (THAA) were determined and quantified on all extract fractions using the method outlined by Cowie and Hedges (1992). Two non-protein amino acid charge-matched standards, α-aminoadipic acid and γ-methylleucine (Fluka), were added to the extract fractions to determine if any amino acids were lost during the hydrolysis procedure. Samples were heated to 150 °C for 70 min with 1 ml of 6 N HCl sealed under N_2. After the hydrolysis, samples were taken to dryness on a roto-evaporator (8 h) and resuspended in 1 ml 0.1 N borate buffer (pH=10). This was repeated until the sample's pH was between 8.5 and 9.5. Prior to separation on a reverse phase C-18 HPLC column (5 μm×4.6 mm×15 cm; Beckman), amino acids were derivatized with *o*-pthaldialdehyde (OPA) (Sigma Aldrich). Glacial acetic acid was added (2 μl) to stop derivitization, stabilize products, increase peak sharpness, and decrease background noise. *O*-methyl-threonine (Fluka) was used as an internal running standard to help quantify between run variability. Separations and quantifications of fluorescent OPA derivatives were conducted as described in Keil et al. (1998) on a Shimadzu HPLC (328 nm excitation and >450 nm emission). Every 40–80 samples, the C-18 guard column was changed. Of the 20 common protein amino acids, 16 were monitored with this method, along with 2 non-protein amino acids (γ-aba, β-ala). All samples were analyzed in duplicate. In order to

ensure that the methods we tested did not hydrolyze the proteins and peptides, and to quantify the contribution of free amino acids removed by the different solvents, splits of each extract were analyzed for amino acids prior to the HCl-hydrolysis (dissolved free amino acids; DFAA). To remain uniform and be able to relate extraction efficiencies, concentrations of total hydrolyzable amino acids are reported as a fraction of Puget Sound whole sediment total hydrolyzable amino acids (*PS*-THAA).

For extraction experiments that involved more than one hydrolysis or experiments where hydrolysis preceded a solubilization procedure, charge match standards were not added. Instead, Puget Sound sediment and Lake Washington standard mud were run in series to determine if there were any amino acids lost during hydrolysis or analysis. Samples were compared and corrected accordingly.

In order to evaluate compositional differences in the amino acids extracted using the six different techniques and relate them to the composition of the whole sediment hydrolysis technique, the Dauwe et al. (1999) degradation index (DI) was applied to the amino acid compositions. The DI was originally developed from a principle component analysis (PCA) of the amino acid compositions of a variety of marine particulate materials ranging from fresh phytoplankton to deep-ocean sediments. PCA analyses can simplify large data sets with numerous variables by resolving sample differences into a few factors that are controlling the variability between the suites of data. The factors can then be examined to determine what they represent in the system that is being analyzed. Using a PCA, they developed a quantitative method to relate different sediments across a degradation regime. Essentially, the relative abundance of each amino acid (mole percentage) is multiplied by a factor coefficient that corresponds to that amino acid (see Table 1 and Results and Discussion: Dauwe et al., 1999) and their resulting values are summed into a single degradation index.

We also performed independent principle component analyses using SPSS software on our data to reduce the data set and search for additional structure within the different extraction efficiencies and compositional yields. Independent PCA on our data set did not provide any additional information on controlling factors between the extractions that were not revealed from the raw data and simple compositional plots; therefore, we decided to report the Dauwe degradation index values because they can be cross-compared to previous sedimentary studies and emphasize how the different extraction solvents used can control the amino acid composition and their resulting DIs.

3. Results

Total hydrolyzable amino acids represent 22% of the total nitrogen and 8% of the total carbon in the Puget Sound sample. These values are within the range of total amino acid-C and -N reported for other marine sediments (Aufdenkampe et al., 2001; Columbo et al., 1998; Cowie and Hedges, 1992; Dauwe and Middelburg, 1998; Hedges et al., 1994; Henrichs and Farrington, 1987; Keil et al., 2000). Yields for *PS*-THAA ranged from 3.2 to 4.3 mg gdwt^{-1}, and are within the range observed for coastal marine sediments (Burdige and Martens, 1988; Cowie and Hedges, 1992; Henrichs

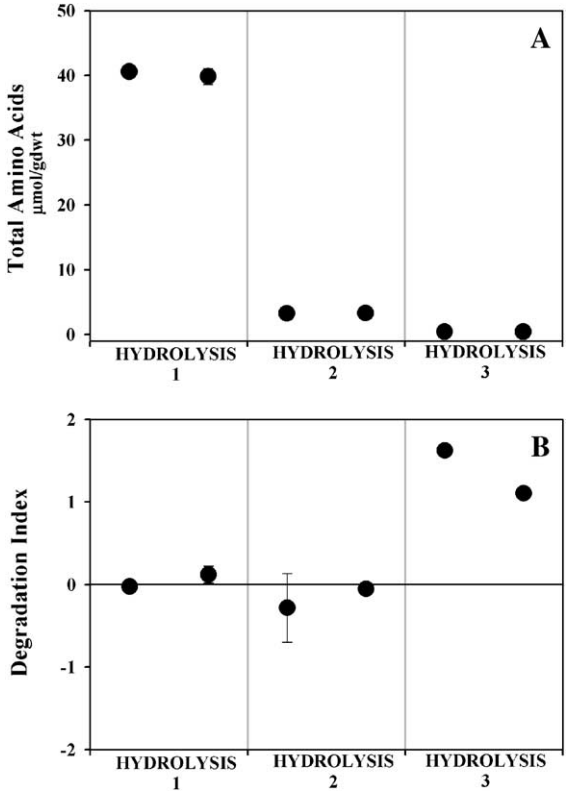

Fig. 1. Sequential hydrolysis. (A) Concentration of total hydrolyzable amino acids per milligram of dry sediment. Duplicate experiments were completed. Dry sediments were hydrolyzed and the soluble fraction was removed (HYDROLYSIS 1). The remaining sediments were hydrolyzed under the same conditions an additional two times (HYDROLYSIS 2 and HYDROLYSIS 3). Between each hydrolysis the soluble fraction was removed prior to the next treatment. (B) Degradation index of THAA, calculated according to Dauwe et al. (1999). Positive values are indicative of 'fresh', more labile amino acid composition, while negative values suggest the sample has experienced degradation.

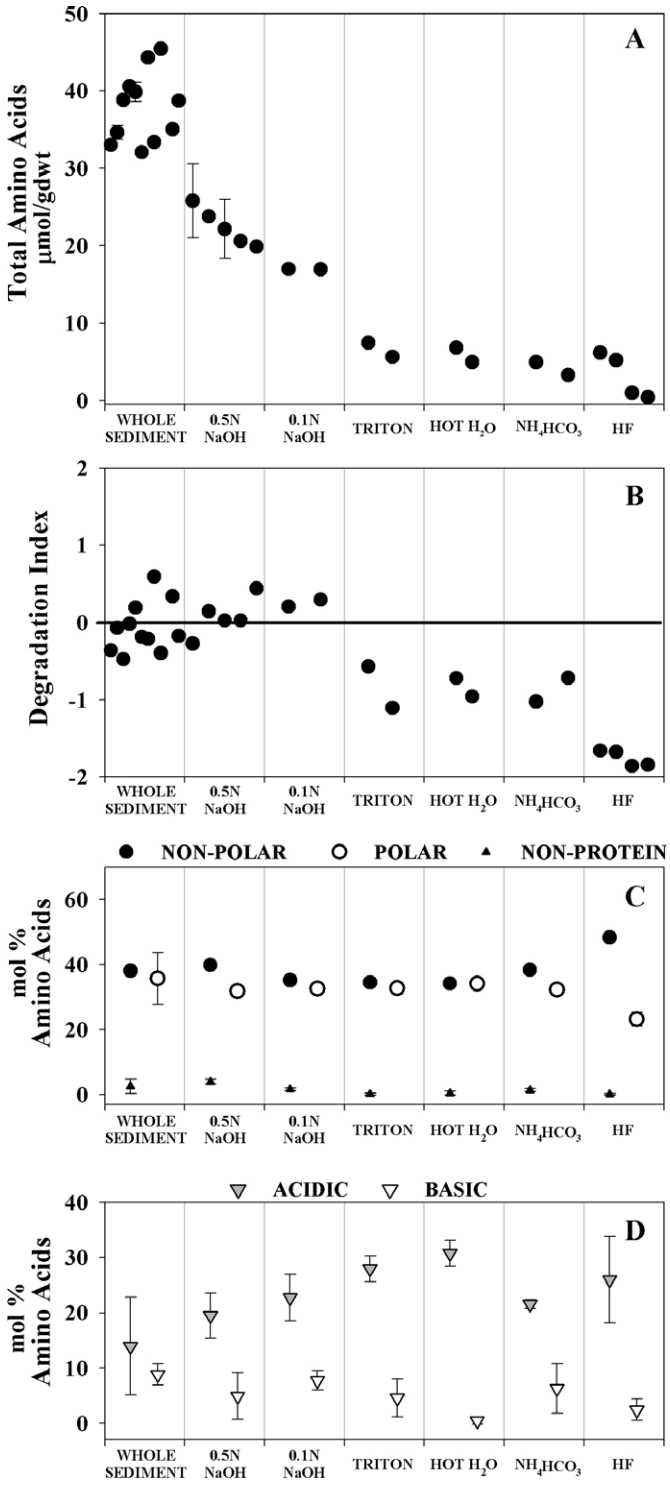

Fig. 2. Extraction experiments. (A) Whole sediment values of THAA ($n=11$) compared to THAA extracted with six different solvents. Multiple experiments were performed and plotted for each method. (B) Degradation index of THAA extracted (Dauwe and Middelburg, 1998). Index values line up with previous graph. (C and D) Average mole percentage amino acid distributions of experiments for each method. Error bars represent standard deviation of the mean. (C) Closed circles represent total mole percent polar amino acids (Gly, Ser, Thr, Tyr); open circles represent total mole percent non-polar amino acids (Ala, Ile, Leu, Met, Phe, Val); solid triangles represent total mole percent non-protein amino acids (α-Aba, γ-Aba); grey triangles represent total mole percent acidic amino acids (Glu, Asp); open triangles represent total mole percent basic amino acids (Arg, His, Lys).

and Farrington, 1987; Keil et al., 2000). The major amino acids observed in these sediments in mole percentages were glycine (22%), alanine (13%), and aspartic acid (9%). The total concentration of dissolved free amino acids (DFAA) in the extracts was <1% of the total hydrolyzable amino acids measured on extracts that were hydrolyzed with HCl (data not shown).

To determine if additional nitrogen could be released as amino acid, the HCl-hydrolysis was repeated twice and amino acids for the sequential hydrolysis were quantified (Fig. 1A). In duplicate experiments, ~92% of the total hydrolyzable amino acids (sum of all three fractions) were released upon the first hydrolysis. In the final two hydrolyses, 7% and 1% of the THAA were released, accounting for only an additional 3% of the TN and 0.8% of the TOC in the sample. The amino acid composition of the first two hydrolyses was very similar. Hydrolysis 3, on the other hand, was enriched in the non-polar amino acids isoleucine and leucine and depleted in glycine. This discrepancy was well illustrated when translated into the Dauwe et al. (1999) degradation index (DI; Fig. 1B). Although the third hydrolysis released a very small percentage of the THAA (~1%), this fraction had a distinctly positive DI signature.

Using a strong base to weaken bonds between organic matter and sediment matrix has been used in studies to investigate proteins, humic acids, melanoidin, and algaenans (Collins et al., 1992; Ferdelman et al., 1991; Francois, 1990; Mayer et al., 1986; Nguyen and Harvey, 1998). The average concentration of hydrolyzable amino acids solubilized with 0.5 N NaOH was 22.5 ± 2.4 μmol gdwt^{-1} ($n=5$), corresponding to 60% of whole sediment *PS*-THAA. Degradation indices (DI) for all five replicates fall within the range calculated from the whole sediment samples (Fig. 2B). Two extractions conducted with a lower concentration of sodium hydroxide (0.1 N NaOH) also yielded degradation indices that agreed with the whole sediments; however, these extractions only solubilized 45% of the *PS*-THAA.

The other extraction methods examined solubilized a small percentage of the total amino acids in the Puget Sound sediment (<16% of *PS*-THAA), each method with distinctly different amino acid compositions, as suggested by an independent PCA and their DI values (Fig. 2A and B). Most commonly, these four methods did not solubilize β-alanine (non-protein) or histidine (basic) at, or above, detectable limits.

Triton X-100 and hot water released ~16% of the THAA of whole sediments and were enriched in aspartic and glutamic acid, depleting the total basic amino acid mole percentages (Fig. 2D). Polar and non-polar amino acid distributions, however, agreed very well with whole sediment values and NaOH extractions.

The NH_4HCO_3 extraction technique, used on acetone-rinsed sediments, solubilized only 11% of the

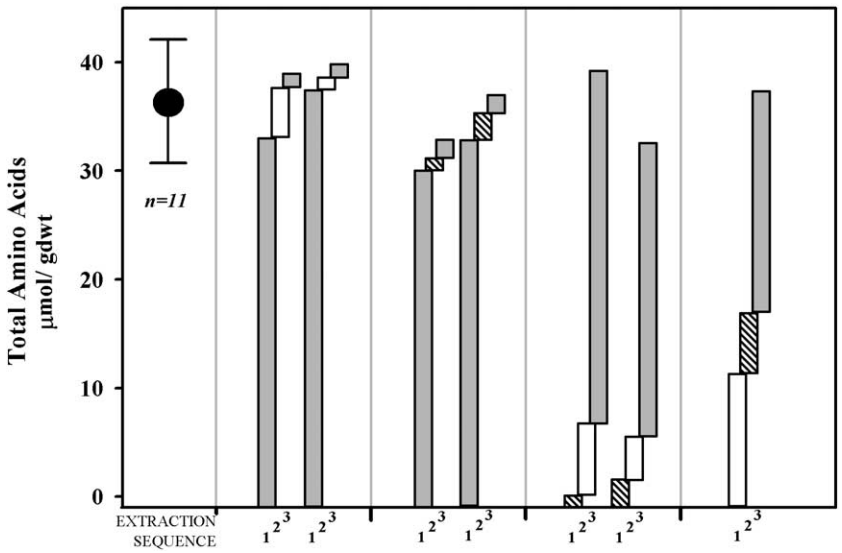

Fig. 3. Extraction combinations. *Y*-axis represents concentrations of total hydrolyzable amino acids per milligram of dry sediment. (●) is the average value of THAA for whole Puget Sound sediments ($n=11$). Error bars are the standard deviation about the mean. Grey bars represent direct hydrolysis on particulate fraction, white bars represent THAA in base soluble fraction (0.5 N NaOH; 2 h at 37 °C), white hatched pattern represents THAA in HF soluble fraction. Four sequential combinations were examined: HYD–BASE–HYD, HYD–HF–HYD, HF–BASE–HYD, and BASE–HF–HYD. Order of treatments are indicated on the *X*-axis. Height of 3 stacked bars is equal to THAA extracted from the sediments per gram dry weight.

THAA with an amino acid distribution similar to whole sediments and NaOH extractions. Undetectable histidine concentrations led to a pronounced negative degradation index relative to whole sediment values. However, low concentrations of valine also drove the index value down. Interestingly, the initial rinses of the sediment with acetone prior to NH_4HCO_3 solubilization recovered 8% of the *PS*-THAA and were heavily enriched in glycine (58 mol%).

Hydrofluoric acid solubilized the lowest percentage of total amino acids (9%) with the most distinct amino acid signature (Fig. 2B). The organic material released by HF solubilization of the sediments was enriched in the polar amino acids glycine (32 mol%) and serine (16 mol%) and depleted in the non-polar amino acids phenylalanine, isoleucine and leucine. Both the enrichment in polars and depletion in non-polars produce a very negative degradation index (−1.7).

For all of the four sequential extraction combinations tested (HYD–BASE–HYD, HYD–HF–HYD, HF–BASE–HYD, BASE–HF–HYD), the first acid hydrolysis on the particulate material liberated the greatest percentage of amino acids, independent of what other extractions were performed first (Fig. 3). When amino acids in the three fractions were summed, none of the sequential combinations released more total amino acids than a single whole sediment hydrolysis. Dissolving the silica matrix with HF prior to solubilization of the amino acids by base (HF–BASE–HYD) did not improve the total yield of extractable amino acid nitrogen.

4. Discussion

The primary objectives of this study were to determine which non-destructive method solubilized the greatest percentage of THAA from coastal marine sediments, and to then evaluate whether the materials extracted using different techniques had different amino acid compositions. Current understanding of protein degradation and preservation in marine sediments has been slowed by analytical limitations such as the necessity to analyze trends in the quantity and distributions of polypeptides only after extracting and hydrolyzing them to amino acids (Keil et al., 2000). Although amino acid trends have helped us decipher some aspects of degradation and diagenesis, knowledge of the specific proteins or peptides preserved in the sediments may provide us with detailed information on their nutritional value, mechanisms of preservation and degradation, and an understanding of bacterial foraging or enzymatic limitations or preferences (Mayer et al., 1995; Nunn et al., 2003). Rapid development in the field of environmental proteomics have proven the capability to investigate a variety of intact proteins >3 kDa isolated from complex environmental matrices (Nunn et al., 2003; Ostrom et al., 2000; Tanoue, 1995; Whitelegge et al., 1998; Yamada et al., 2000). Analysis of protein in coastal sediments is particularly difficult because these sediments are rich in other organic matter that can interfere with extraction and analysis. Additionally, it is uncertain whether coastal sediments are dominated by a few preserved proteins or several thousand (Nguyen and Harvey, 1998; Tanoue, 1995). In order to appropriately analyze and identify environmentally preserved proteins in sedimentary systems, baseline knowledge of the efficiency of extraction techniques must be established and a reproducible non-destructive method of extraction needs to be identified.

Total hydrolyzable amino acids in Puget Sound sediments accounted for 22% of the total nitrogen, which is on the low end of values that have been previously reported for marine sediments (THAA-N/TN=8–50%) (Burdige and Martens, 1988; Henrichs et al., 1984; Keil, 1999; Lee and Cronin, 1982; Wakeham et al., 1997). To evaluate methodological reproducibility and sample variability, 11 splits of the Puget Sound sediment were hydrolyzed, ranging in weight from 4 to 50 mg dry weight. The average concentration of total amino acids was 3.52 mg $gdwt^{-1}$ +10% (SMD), similar to THAA values recovered by the identical procedure in other Puget Sound marine sediments (6 mg $gdwt^{-1}$ +4% SMD) (Cowie and Hedges, 1992).

4.1. Sequential hydrolyses

To determine if the HCl-hydrolysis was effective at removing the majority of amino acids, the HCl-hydrolysis procedure was repeated twice more on the same sediment samples and each fraction's amino acids were quantified. Subsequent hydrolyses on the same sediment did not release a large fraction of total amino acids. The second hydrolysis appears to have further released amino acids from the same components that the first hydrolysis attacked. Mole percentages for 14 of the 18 different amino acids are not significantly different; the exceptions are two of the non-protein amino acids β-alanine and α-aminobutyric acid, and polar amino acids serine and threonine. To recover equivalent non-protein mole percentages as the first hydrolysis, concentrations for β-alanine and α-aminobutyric acid would have been 0.02 and 0.004 μmol $gdwt^{-1}$, concentrations undetectable by the amino acid analysis we

employed (Cowie and Hedges, 1992). The discrepancy in concentrations of serine and threonine is the result of poor chromatographic resolution for these nearly co-eluting components. If we attribute the minor differences in amino acid distributions to be a result of analytical capabilities, the second hydrolysis looks identical to the first hydrolysis. In contrast, the third hydrolysis extracted material enriched in isoleucine and leucine and greatly depleted in glycine. We investigated the unique distribution of amino acids in Hydrolysis 3 by looking at the relative hydrophobicity of the different amino acids. Four hydrophobicity scales were used: two rank the amino acids based on the physiochemistry of the side chains, and two scales examine proteins with known 3-D structures and rank residues based on their tendency to be found inside the protein, rather than on the surface (Janin, 1979; Kyte and Doolite, 1982; Rose et al., 1985; Wolfenden et al., 1981). All of these hydrophobicity scales ranked isoleucine and leucine as two of the most hydrophobic amino acids. This suggests that, although it is a small fraction (~1% of the THAA), the third hydrolysis extracts material from a distinct component. This component may be diatom biomass. When the third hydrolysate was compared to the average amino acid mole percentages present in cultures of the diatoms *C. gracilis*, *C. gradiatus*, and *T.pseudonana* (Ingalls et al., 2003), no significant differences were observed (Fig. 4). Thus, once the majority of amino acids are removed via hydrolysis steps 1 and 2, the third hydrolysis may be successfully extracting well-protected phytoplankton amino acids or proteins. The size of this component is likely to change in an area dominated by planktonic contributions rather than terrestrial input.

4.2. Extraction efficiency

There are several studies that have examined solubilization of amino acids from particulate matter and sediments (Cowie and Hedges, 1992; Henrichs and Farrington, 1987; Ingalls et al., 2003; Keil and Fogel, 2001; Mayer et al., 1986; Nguyen and Harvey, 1998; Pantoja and Lee, 1999). As a result, several different fractions of extractable amino acids have been defined and have earned their own acronym (e.g., THAA, DFAA, EHAA, SiTHAA, DCAA, and TEAA). Our objective was not to isolate a particular size class of an adsorbed or trapped population of amino acids, but instead to determine which of the commonly used methods liberated the greatest quantity of organic matter in the most reproducible manner. Our investigation also allows determination of the amino acid composition of recovered materials, which is timely because interest in extracting and characterizing the sedimentary protein component is rising.

Extractions with 0.5 N NaOH consistently solubilized the greatest fraction of amino acids (Fig. 2). Henrichs and Sugai (1993) used NaOH to solubilize ^{14}C-labeled proteins adsorbed to sediments and compared the extractions to an ion exchange solution ($Na_4P_2O_7$) and an acid (0.5 N HCl); they also concluded that extracting with a base was the most effective and consistent means of solubilizing amino acids with different functional groups. Strong base likely displaces most proteins and amino acids from the negatively charged binding sites on the particle surface, making it the most efficient extracting agent. Mayer et al. (1986) demonstrated that longer and/or multiple extractions with NaOH did not release any more amino acids.

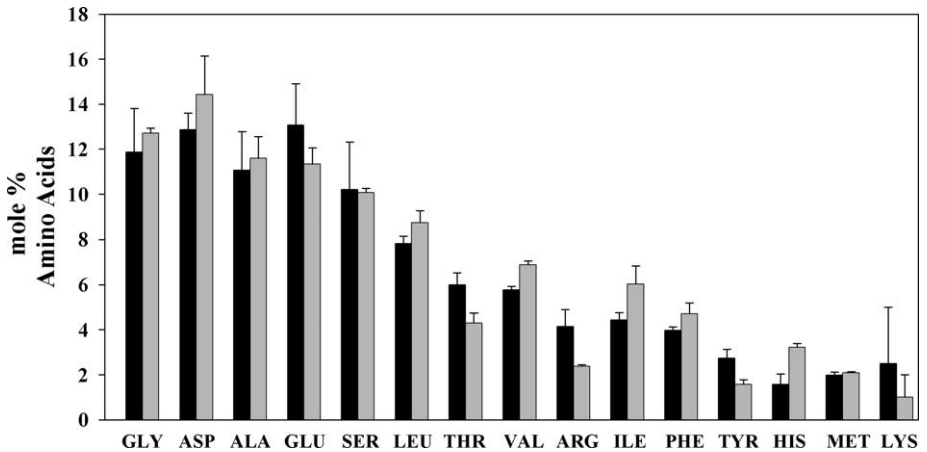

Fig. 4. Comparison of THAA extracted during HYDROLYSIS 3 and THAA of total biomass from 3 cultured diatoms. *Y*-axis is in mole percent of each amino acid (total=100%). Black bars represent THAA in third hydrolysis of particulates. Height of bar represents average of duplicate analyses. Dark grey bars represent average THAA of total biomass from three species of cultured diatoms (Ingalls et al., 2003).

The extractions with hot water and Triton X-100 removed low yields of the total hydrolyzable amino acids in Puget Sound sediments (16% of *PS*-THAA; Fig. 2A). Although the overall percent recovery of amino acids was low for these two extraction techniques, they were both enriched in the acidic amino acids. Glutamic acid and aspartic acid are both very hydrophilic and tend to be found on the surface of proteins, rather than the interior (Kyte and Doolite, 1982; Wolfenden et al., 1981). Since it is likely that these proteins are large, flexible chains of amino acids, hydrophilic loops or tails of peptides are probably not attached to the sediment surface, but instead are in solution. This would suggest that hydrophobic domains are therefore bound to the mineral and are relatively protected from extraction or potential enzymatic attack (Aguilar et al., 1998; Mayer et al., 1995; Nunn et al., 2003). Hot water or Triton X-100 extractions might preferentially be liberating the more hydrophilic components suspended in solution. These two methods would therefore provide a novel way of examining the hydrophilic domains available for enzymatic attack (Mayer et al., 1995) or the 'protected' hydrophobic domains of the adsorbed proteins.

Nguyen and Harvey (1998) take a novel approach at extracting protein by first solubilizing and removing smaller 'undesirable' organic components from the sediment. This was accomplished by rinsing the sediments three times with an acetone–TCA mixture at −20 °C. Both cold acetone and TCA have been used frequently by biochemists for the precipitation of proteins by changing the conformation to a tightly condensed form (Wei et al., 2003). The acetone will also solubilize other organic matter that is not large enough to precipitate out. Keil and Kirchman (1991) suggested that the cutoff for this type of precipitation is approximately 2 kDa for proteins. The remaining larger protein-based material was solubilized into a mild basic (NH_4HCO_3) buffer. On diatom-rich Mangrove Lake surface sediments, Nguyen and Harvey (1997) reported that nearly 80% of the THAAs were extractable with this method, whereas <20% were solubilized in the 4-ky-old sediments. Our investigation of this method on coastal sediments suggested that 11% of the THAA were extractable and involved in proteinaceous material >2 kDa. This is only slightly larger than the amount of amino acid released in the rinsing steps (8% of *PS*-THAA). Assuming the rinsing step with the TCA precipitation does reliably remove organic matter <2 kDa, results would suggest that ~8% of the *PS*-THAA in these coastal sediments are degraded fragments <2 kDa and enriched in glycine. Glycine is thought to have little nutritional value because of the absence of a side chain (–H) and might be preferentially ignored by enzymes (Dauwe and Middelburg, 1998; Nunn and Keil, 2005), consequently enrichments can be inferred to be an indicator of highly degraded organic matter or the presence of diatom frustule material (Hecky et al., 1973; Ingalls et al., 2003). Sediments remaining after the acetone-rinsed NH_4HCO_3 extraction contained 81% of the *PS*-THAA in whole sediments, and had a degradation index similar to whole sediment values. Protein components >2 kDa likely remain in this fraction, but were not solubilized at such a neutral pH (7.8).

Hydrofluoric acid releases the organic matter from sediments by chemically dissolving the matrix. It has the ability to decompose silica-based clay particles without seriously modifying most organic matter (Bremner and Harada, 1959). The method has been used to solubilize and remove the mineral matrix in sediments to prepare the remaining organic matter for analysis by NMR (Gelinas et al., 2001). In 1959, Bremner and Harada utilized this same silica dissolution to examine the potential of HF to release organic matter bound to the matrix into solution. Although Bremner and Harada (1959) were unsuccessful at releasing a significant fraction of the soil total organic carbon (TOC), Durand and Nicaise (1980) treated recently deposited marine sediments with HF and solubilized over 50% of the TOC. Treatments with 100% HF are also frequently used to solubilize polyamines and peptides bound within diatom silica frustules for mass isolation and purification (Ingalls et al., 2003; Kröger et al., 1999). We were interested in determining if HF released a fraction of organic matter bound to or within the silica matrix of near-shore coastal marine sediment and if this trapped component had a unique signature (e.g., silica cell-wall bound or trapped proteins). The HF soluble fraction of the Puget Sound sediment, however, represented a low percentage of both TN (<3%) and TOC (~1%), very similar to values found by Gelinas et al. (2001). Amino acids present in this fraction were not similar to those in whole diatom cultures (Ingalls et al., 2003), however they did display some of the key amino acid characteristics observed in clean diatom frustules (Hecky et al., 1973; Ingalls et al., 2003). Although the mole percentages do not compare exactly, the general similarities include enrichments in glycine and serine plus threonine and depletions in aromatic amino acids (phenylalanine) and acidic amino acids (glutamic acid and aspartic acid). Particulate residue left over from the demineralization procedure did not have significantly different mole percentages of the different amino acids than the

whole sediments. Subsequent extractions on the demineralized sediment residue were carried out with NaOH and did not show improved recovery of sedimentary nitrogen (Fig. 3) (Bremner and Harada, 1959). In fact, none of the combinations of extractions or pre-treatments to sediment improved the extraction efficiency of sedimentary nitrogen (Fig. 3). These experiments provide evidence in support of the theory that a significant fraction of the nitrogen in sediments remains trapped within an organic matrix (Nguyen and Harvey, 2001).

Including acid hydrolysis, the various techniques evaluated extract only 2% to 22% of the total sedimentary nitrogen as THAA. Strong base (0.5 N NaOH) is the most efficient method for solubilizing organic matter from a mineral matrix without hydrolyzing the macromolecular components. Based on both degradation index by Dauwe et al. (1999) (or independent PCA analyses) and raw amino acid compositional data, the base extractions solubilize the protein-component most representative to the whole sediment. Of the two concentrations examined, 0.1 N and 0.5 N NaOH, the stronger concentration of base extracted the greater percentage of the sedimentary TN as THAA (~12% of TN in Puget Sound sediment). Sodium hydroxide may be more rigorous at releasing organic matter from mineral matrices than the other methods examined because it both actively solubilizes the biogenic silicate matrix (Ragueneau and Treguer, 1994) and it denatures the proteins (Voet and Voet, 1990), which may result in protein unfolding and weakening bonds between proteins and silicates. Although sodium hydroxide can dramatically change the stability and conformation of most proteins adsorbed onto sediments, it is unlikely to hydrolyze them under such mild conditions for such a short period of time. DFAA analyses on extractions indicated that <1% of the amino acids released are free amino acids (results not shown). More extreme temperatures and/or stronger concentrations of the alkali might solubilize more of the TN from the sediments; however, such conditions are likely to break peptide bonds and misrepresent the sizes of the biomolecules preserved in the sediments (Kaplan et al., 1998; Wakim and Aswad, 1994). One of the primary arguments against the use of alkali reagents for solubilization of organic matter from sediments is that it likely changes the 'natural' conformation (i.e., tertiary structure) of the molecules (Bremner and Harada, 1959; Mayer et al., 1995). This is a valid concern; alkali extractions are not likely useful in elucidating original conformational structures or folding properties of the biomolecules adsorbed to the sediments. However, base extraction appears to be the best approach available for isolating material prior to mass spectral attempts to determine the molecular weights and the amino acid sequence of preserved proteins or peptides (i.e., secondary structure). Extractions with hot water or Triton X-100 may provide useful information on specific domains of adsorbed proteins, but do not release a large fraction of the material present. To consistently release the largest fraction of total sedimentary organic nitrogen from the sediments, we recommend treating the sediments with 0.5 N NaOH at 37 °C for 2 h. This extract can then be subjected to one or several of the different chromatographic or precipitation methods for isolation and separation of different proteins (Gobom et al., 1999; Kaufmann, 1997; Kussmann et al., 1998).

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