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Hydrolysis patterns and the production of peptide intermediates during protein degradation in marine systems

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Abstract

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was used to evaluate the degradation of the protein bovine serum albumin (BSA) added to seawater. The production of peptides during degradation, the size of the peptides produced and the within-protein locations of protease attack were all monitored in an effort to evaluate whether specific types of proteases or specific peptide bond locations were targeted during BSA degradation. Analysis of products from the bacterial degradation of proteins in both seawater and sediment pore water revealed the creation and release of peptide intermediates into the medium. Peptides observed in seawater degradation experiments were all less than 40 amino acids long, whereas sedimentary digestions of the same protein generated peptide fragments in the pore water ranging from 110 to 508 amino acids in length. Neither of the environments demonstrated recurring cleavages adjacent to one amino acid or functional group that might be indicative of the action of a single protease. Instead, it appears that a mixture of bacterial proteases were involved in the degradation of protein. The MALDI-TOF-MS method presented, in combination with these results, can help us delineate some of the processes responsible in the initial stages of degradation and better understand how proteins are partitioned between dissolved and solid phases in marine systems.

Keywords: MALDI-TOF-MS; Protein; Degradation; Sedimentary organic matter; Dissolved organic matter; Pore water

1. Introduction

Studies of protein and peptide dynamics in seawater and in marine sediments are relatively common, and many parameters have been evaluated including amino acid concentrations and compositions, protein and/or amino acid degradation rates, protease hydrolysis rates and the enzymatic availability of hydrolysable amino acids to heterotrophic organisms (see: Henrichs, 1992; Keil et al., 2000; Wakeham and Lee, 1989 for reviews). Perhaps the greatest limitation preventing better understanding of protein cycling in marine environments is the inability to specifically identify the proteins and peptides present in a sample. For example, while marine scientists are adept at evaluating the concentrations, amino acid compositions, and degradation rates of proteins in marine systems, there remains very little knowledge of the specific proteins present in marine systems or the enzymatic methods used by marine microorganisms to facilitate protein utilization. This is a difficult task, as marine waters and sediments contain many different proteins and peptides present at various concentrations and stages of degradation, and

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many may be chelated or conjugated with other organic compounds, metals or minerals.

Mass spectrometry offers the potential to evaluate protein mixtures in environmental samples. Advances in sample ionization technologies have greatly facilitated the evaluation of proteins within complex (e.g., salty) matrices. One technique that is currently available is matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) because it is a technique that allows ionization of intact peptides and proteins and subsequent mass resolution. MALDI-TOF-MS utilizes a soft ionization that provides the mass-to-charge (m/z) identification of large biomolecules. Less than 1 μl of the analyte is dried with an equivalent volume of concentrated acidic matrix solution on a metal target. While drying, the compounds of interest become embedded into the crystalline matrix. The analyte–matrix target is then ablated with a pulsing laser, desorbing and ionizing clusters of the analyte, while the matrix absorbs much of the photon energy. This temporally defined group of ions is subjected to an electric field, accelerating them toward the detector through a field-free flight tube. Each ion will cover the flight tube distance in a time that depends on their mass-to-charge ratio. Unlike many previous attempts to ionize large biomolecules, MALDI-TOF-MS almost always desorbs and ionizes the analyte unfragmented and singly charged (see Overberg et al., 1992; Zenobi and Knochenmuss, 1998).

Currently in our laboratory, we use MALDI-TOF-MS to evaluate the proteins and peptides present in marine systems and to assess the manner in which proteins are degraded within marine systems. In this paper, we describe the use of MALDI-TOF-MS to examine mass spectral fingerprints obtained during the microbial degradation of a known protein, bovine serum albumin, in seawater and in a marine sediment slurry. With these fingerprints, we can determine exactly where the original protein was cleaved, thus elucidating hydrolysis patterns.

Numerous models describing proteolysis or uptake of proteins and peptides by bacteria exist. The most accepted model suggests that extracellular enzymes are released to hydrolyze proteinaceous material, and the resulting components are either transported to the cell membrane or diffuse away into the environment, available for other organisms to utilize (Mayer et al., 1995; Vetter, 1998). Peptide intermediates created during the degradation of proteinaceous material may or may not be present in marine environments, depending on whether the protease cleaving the protein is cell associated or freely dissolved in the water. During degradation experiments, Hollibaugh and Azam (1983) did not observe the presence of intermediate peptides between the starting protein, bovine serum albumin (BSA) (66,480 Da), and the final products (< 700 Da). More recently, both Keil and Kirchman (1991) and Pantoja and Lee (1999) observed the release and utilization of intermediate low-molecular-weight counterparts from starting proteins and peptides. Complete degradation of proteins appears to be a stepwise process where hydrolysis rates depend on the size of the peptide substrate (Pantoja and Lee, 1999). The work of Cottrell and Kirchman (2000) supports this theory of communal foraging. Their experiments demonstrated that *Cytophaga–Flavobacter* bacteria preferentially consume high-molecular-weight dissolved organic matter (DOM), whereas α-*Proteobacteria* favor amino acids. These studies suggest that a diverse assemblage of bacteria is required for complete degradation of complex-dissolved organic matter in the ocean.

Most enzymes excreted by bacteria to hydrolyze macromolecules are thought to cleave specific chemical linkages (Billen, 1991; Rogers, 1961). As a result, the chemical structure of a protein is an important factor when trying to decipher what controls its degradation or preservation in the marine environment. The goal of our present study was to investigate bacterial degradation of a known protein in a marine system and to focus on the patterns of cleavage and the creation of peptides. Rather than looking at the natural proteins present in a sample, we have spiked a marine environment with a known protein to monitor the chemical structures of the beginning and end products. Here, we describe the application of MALDI-TOF-MS to marine systems, quantitatively analyze peptide creation and degradation, and qualitatively analyze the peptide intermediates produced.

2. Methods

2.1. Degradation experiments

Surface seawater and sediment samples were collected from a water depth of 250 m in October 2001

from central Puget Sound (WA, USA) while aboard the *R/V Clifford A. Barnes*. For the seawater degradation experiments, unfiltered seawater was placed in a 2-l container, subsampled and then spiked with bovine serum albumin (BSA) from Sigma-Aldrich to a final concentration of 1 μM. To increase solubility of proteins used throughout these experiments, 1–2 μl of analytical grade 10% HCl was added per milliliter of water. The seawater experiment was conducted in a dark coldroom held at ambient Puget Sound seawater temperature (8 °C) and sampled over a 12-h period. Samples were filtered though combusted GF/F filters, and then 10 ml was flash frozen and stored in muffled glass vials until analysis. All MALDI-TOF-MS analyses were conducted within 48 h of sample collection.

Sediments were collected using a box corer and immediately sieved to remove macrofauna, homogenized by stirring with a Teflon rod and centrifuged to separate the pore water from the solid phase (18,000 × *g*, 15 min). UV-oxidized seawater was added to these centrifuged sediments, and an initial sample of the slurry was taken. A BSA spike was prepared by dissolving BSA in UV-oxidized seawater plus a few drops 10% HCl. The BSA solution was then added, and the sediments were slurried with the new 'artificial' pore water (UV-oxidized seawater) (Arnarson and Keil, 2000). The sediment concentration was 166 $gdwl^{-1}$ (equivalent to porosity of 0.94), and the initial BSA concentration was 68 μM. The slurry experiment was conducted in a dark coldroom (8 °C) for 72 h. At each time point, 2-ml samples were taken, with more frequent sampling at the beginning of the experiment. Once taken, samples were centrifuged to remove the pore water (18,000 × *g*, 15 min). Both sediment and pore water were flash frozen and stored in glass containers until analysis.

To extract proteins from the solid phase, the protocol of Nguyen and Harvey (2001) was used. This method avoids the common use of a strong base and high temperatures for protein extraction. Cold trichloroacetic acid (TCA; Sigma-Aldrich) is used to precipitate the proteins, and an acetone/β-mercaptoethanol mixture is used to solubilize biochemicals that might interfere with the analysis of protein. The final step involves solubilizing the protein in 2 ml of 37 °C sodium phosphate buffer (pH 7.4) for 24 h. This solution was partially desalted and concentrated by adding an equal volume of distilled water and processing through a micro-ultrafiltering unit (MW cutoff of 3000 Da, Amicon/ Millipore, 5000 × *g*, 150 min, 4 °C). The final solution (~ 100 μl) was resuspended in distilled water to a final volume of 2 ml and retained for determinations of protein concentration.

2.2. Bulk protein analysis

Protein concentrations were measured in duplicate using Coomassie Blue dye (Bradford, 1976; Setchell, 1981). Coomassie Blue (CB) binds to arginine via anion attraction and is sensitive only to larger polypeptides ranging from 10 to 25 amino acid residues (Bradford, 1976). The CB microassay technique works within a sensitivity range of ~ 1–1500 μg of protein ml^{-1}; therefore, sediment solid phase and pore water samples were diluted accordingly (up to a factor of 5) to fall within the readable range. We used a complimentary method to cross-check the protein concentrations in the sediments, CBQCA, since humic materials have been observed to interfere with Coomassie Blue (Mayer et al., 1986). The CBQCA assay from Molecular Probes (http://www.molecularprobes.com) has never been applied (to our knowledge) to marine samples. The reagent ATTO-TAG-CBQCA (3-(4-carboxybenzoyl) quinoline-2-carboxaldehyde) reacts with primary amines in the presence of cyanide, yielding a highly fluorescent product. Low-molecular-weight polypeptides and high-molecular-weight proteins can be quantified. The effective range for the assay is 10 ng ml^{-1}–150 μg ml^{-1}, and it is compatible with many lipids and detergents. A list of salts and organics that react with this product can be found at the Molecular Probes website. In this paper, we do not investigate the ability of this probe to analyze natural protein and peptide concentrations present in marine samples. A series of analyses would need to be carried out, for example, to look at effects of humic acids or natural NH_3 concentrations that might interfere with the fluorescent probe. Because the sedimentary experiment was spiked with BSA (final concentration 4.48 mg ml^{-1}) and the detection limits for protein with this technique are low, samples and standards (made in artificial seawater) were diluted with nanopure water by a factor of 10. This reduced protein concentrations to a range readable by the fluorometer. Fluorescence

emission at 550 nm was measured on a TD 700 Turner Laboratory Fluorometer with an excitation wavelength of 465 nm.

2.3. MALDI-TOF-MS analysis

Initially, to investigate the use of MALDI-TOF-MS in proteomic studies in marine environments, we examined mass spectra of protein mixtures and monitored enzymatic digests of a known protein in seawater. A critical step in the MALDI-TOF-MS technique is the embedding of the analyte into the matrix. The fundamentals of laser ionization and desorption of the matrix and analyte are not fully known (Zenobi and Knochenmuss, 1998). However, experimental evidence suggests that as the analyte dries with the matrix solution, the molecules of interest become isolated from one another in the crystalline lattice. This is essential because as the laser is applied, the matrix assists in ion formation by absorbing and evenly distributing the energy (Karas et al., 2000; Zenobi and Knochenmuss, 1998). Several matrices are commercially available and synthetically made, each yielding different performance results. We tested three of the most commonly used matrices used for large biomolecules: α-cyano-4-hydroxycinnamic acid (HCCA, Sigma-Aldrich), sinapinic acid (SA, Fluka) and 2,5-dihydroxybenzoic acid (DHB, Aldrich). Matrix solutions were prepared by supersaturating a solution of 0.1% TCA in 40% acetonitrile with the dry matrix crystals. The solution was then centrifuged to precipitate out any undissolved crystals. Samples were prepared on target using the dried droplet method (Kussmann et al., 1998). Approximately 0.7 μl volume of both the sample and the matrix were deposited and mixed on the target and then allowed to dry in the ambient air. Mixing the analyte and matrix prior to depositing on the target was also evaluated along with the commonly used sandwich and thin-layer method (Kussmann et al., 1998). The dried droplet method conserved analyte and yielded a high crystal density with well-dispersed proteins. DHB was the best all-around matrix we evaluated for peptide and protein analysis in seawater samples. Sinipinic acid showed promise for the analysis of low-molecular-weight peptides in seawater; however, here, we present only the spectra produced from samples mixed with DHB.

MALDI mass spectra were recorded on a Bruker Reflex III TOF mass spectrometer (Bruker-Franzen, Bremen, Germany) with a single probe inlet, equipped with a UV-nitrogen laser. All spectra of compounds >8000 Da were acquired in positive-ion linear mode, while spectra of compounds <8000 Da were acquired in positive-ion reflection mode, and the X-mass data system was used for spectra acquisition and instrument control. Typically, 100–500 laser shots were accumulated in the resulting spectra, and attenuation was approximately 39%. Most spectra were collected by ablating along the outer rim of the target. Mass calibration was performed using both the singly and doubly protonated molecular ion signals of the BSA standard.

To evaluate mass accuracy and sensitivity of MALDI-TOF-MS to proteins of various masses, single proteins and protein mixtures were dissolved in GF/F-filtered, UV-oxidized seawater. A mixed protein standard was made with aprotin, trypsinogen, myoglobin and BSA (all purchased from Sigma-Aldrich). These proteins ranged in size from 7000 to 70,000 Da.

The enzymatic digest of bovine serum albumin was carried out using trypsin (Promega, Madison, WI), a protease that cleaves specifically at the C-terminal side of arginine (R) and lysine (K). As both arginine and lysine are abundant in many proteins including BSA, numerous fragments of predictable size and sequence are produced during a digest. By digesting a known protein with trypsin, we were able to check fragmentation patterns and mass accuracy of the MALDI technique on seawater samples. BSA was dissolved into GF/F-filtered, UV-oxidized seawater with 1–2 μl of 10% HCl to a final concentration of 1.5 μM. After an initial sample was taken (T_o), trypsin, resuspended in 50 mM NH_4HCO_3 reaction buffer, was added (trypsin/BSA = 1:100). Digests were incubated at 37 °C for 24 h and 100-μl subsamples were removed through time. To stop enzymatic reactions, 5 μl of glacial acetic acid was added. Mass analysis was done on the proteolytic fragments using MALDI-TOF-MS as described above.

To confirm our mass analyses of both the trypsin digest and the marine bacterial degradation of BSA, we employed three internet-based protein identification software systems: PeptideMass, FindPept and PeptIdent. PeptideMass artificially cleaves a user-

defined protein (e.g., BSA) with a chosen enzyme (e.g., trypsin) and computes the masses of the generated peptides (http://ca.expasy.org/tools/peptide-mass.html). FindPept identifies experimentally determined peptide masses from unspecific cleavages of a known protein (http://ca.expasy.org/tools/findpept.html). It compares measured peptide masses to all possible theoretical peptide masses that could be created from the initial protein. This allows the user to view all possible cleavage points on the original protein that would produce the masses experimentally observed. For the present study, the *m*/*z* ratios for all detected ions were assumed to represent singly charged ions, and 0.03% tolerance was allowed in the database searches. Finally, PeptIdent allows the user to specify the enzyme used for the digestion of an 'unknown' protein (http://ca.expasy.org/tools/peptident.html). That is, PeptIdent attempts to predict the sequence and identity of an initial protein based on a known enzymatic digest. Users can also specify the species to be searched, the molecular weight of the parent ion and/or the p*I* of the protein. PeptIdent then uses the specified enzyme to calculate theoretical peptide fingerprints from thousands of proteins. It then compares the experimental peptide fingerprint to the theoretical peptide fingerprints, calculates the correlation percent and ranks the possible precursor proteins in order of best 'fit'.

3. Results

3.1. MALDI standards

To determine the utility of the MALDI-TOF-MS technique on large biomolecules, a mixture of standard proteins was analyzed ranging in size from ca. 7000 to 70,000 Da (Fig. 1A). Singly and doubly protonated molecular ion signals were attained for myoglobin, trypsinogen and BSA, while aprotin yielded only the singly charged state. It has been suggested that some proteins and larger peptides are difficult to ionize and can fragment in the ion source region of the MALDI-TOF-MS (Polce et al., 2000). The ionization potential, fragmentation and the mass accuracy measurement of BSA was determined independently (Fig. 1B). Because the $[M+H]^+$ and $[M+2H]^{2+}$ charge states were consistently and easily resolved, BSA was also used to calibrate the instrument. The resolution for MALDI-TOF-MS depends on the quality of ionization of the analyte. We were often able to detect BSA in UV-oxidized seawater using the Bruker Reflex III instrument at concentrations as low as 10–100 nM. However, we note that this 'detection limit' is subject to a variety of factors, and that we were unable to resolve BSA in seawater at such low concentrations using alternative MALDI-TOF-MS instruments.

3.2. Trypsin digest of BSA

MALDI-TOF-MS was used to analyze 0.7-μl aliquots from a tryptic digest of BSA to test for peptide separation and resolution (Fig. 1C). There are 55 potential fragments that can be produced by complete digestion of BSA with trypsin (i.e., no missed cleavage sites). A total of 24 peptide fragments were identified, and their sequences were matched to BSA (Table 1). Twenty-two of those fragments could be assigned to peptides from the primary structure with no missed cleavages (40% of the predicted peptide fragments). The *m*/*z* peaks at 1250 (Phe35–Lys44) and 1441 (Arg360–Arg371) could only be matched to peptides with one missed cleavage site (*R*/F*KD*LGEEHFK/*G* and *R*/*RH*PEYAVSCLLR/*L*). Cumulatively, the peptide fragments resolved on the MALDI-TOF-MS after the 24-h digestion can account for 40% of the entire BSA sequence. These results are typical for controlled trypsin digests of BSA in a variety of solutions and settings; it is rare for trypsin to achieve better than 40% coverage. This indicates that the presence of the seawater did not inhibit our ability to identify BSA degradation products using MADLI-TOF-MS.

3.3. Seawater degradation of BSA

No peptides were observed by MALDI-TOF-MS in the initial, unconcentrated and unamended sample. In the degradation experiment of BSA added to seawater, protein was removed at an initial rate of 2 μM BSA h^{-1}. After 4–6 h, no further protein degradation was observed (Fig. 2A). The overall experiment yielded a first-order decay constant of 0.531 h^{-1}. The BSA parent ion was visible at all experimental time points. Numerous peptide frag-

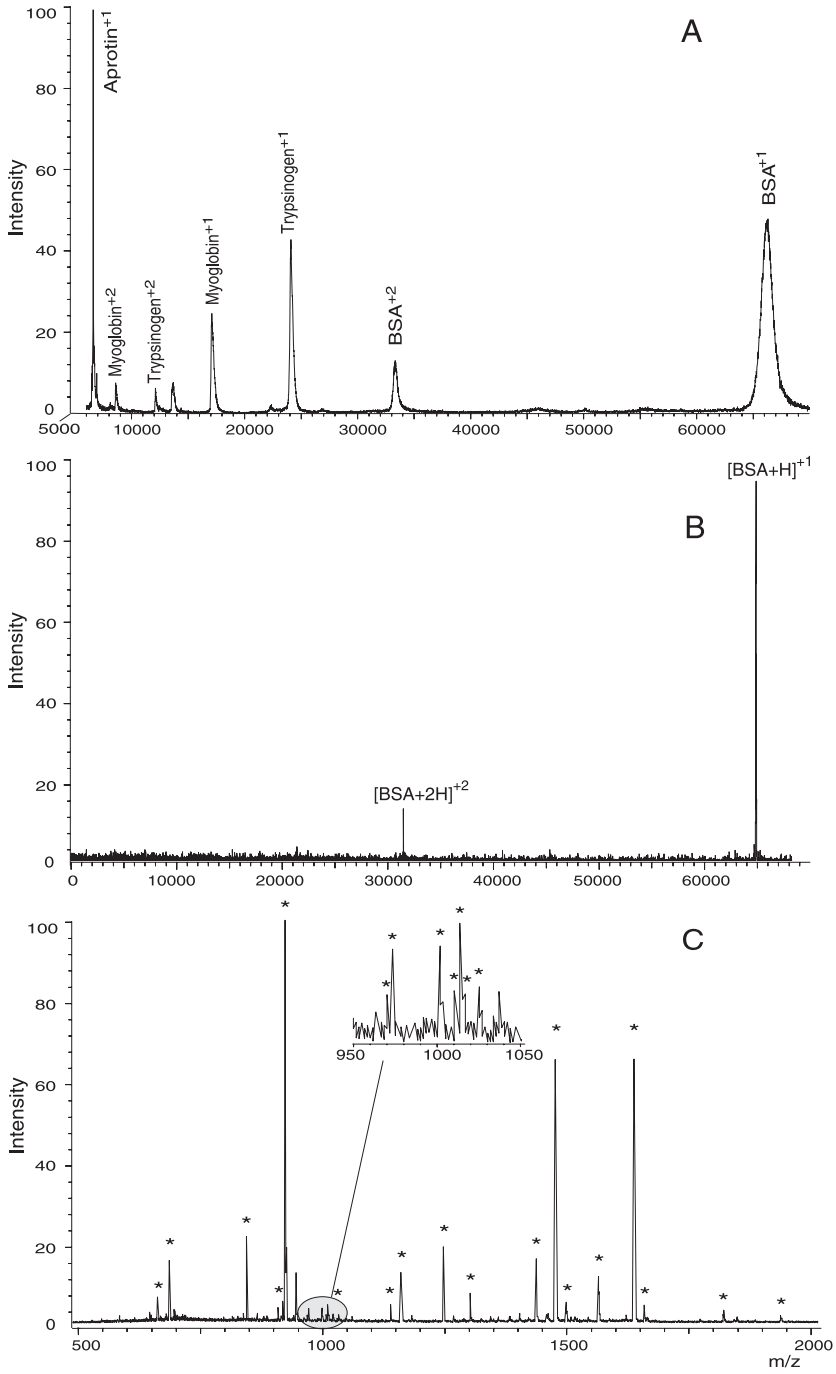


Fig. 1. (A) MALDI-TOF mass spectra of mixed standards: aprotin (6500 Da), myoglobin (16,952 Da), trypsinogen (23,981 Da), BSA (66,480 Da) (B) MALDI-TOF mass spectra of bovine serum albumin 66,480 $[BSA+H]^{+}$ and 33,240 $[BSA+2H]^{2+}$. (C) MALDI-TOF mass spectrometric peptide mapping analysis of tryptic peptide fragments from BSA. Asterisks (*) denote peptides that were matched to calculated peptide fragments from BSA digestion.

Table 1
Peptide mapping analysis of ions observed by MALDI-TOF analysis of trypsin digestion of BSA (Fig. 2b)

$[M+H]^+_{(calc.)}$	Partial sequence[a]
660	490–495
689	236–241
841	483–489
922	249–256
927	161–167
974	37–44
977	123–130
1003	598–607
1011	413–420
1015	549–557
1016	310–318
1024	499–507
1154	146–156
1164	66–75
1250	**35–44**
1306	402–412
1440	**360–371**
1480	421–433
1512	438–451
1568	347–359
1634	184–197
1668	469–482
1824	508–523
1956	319–336

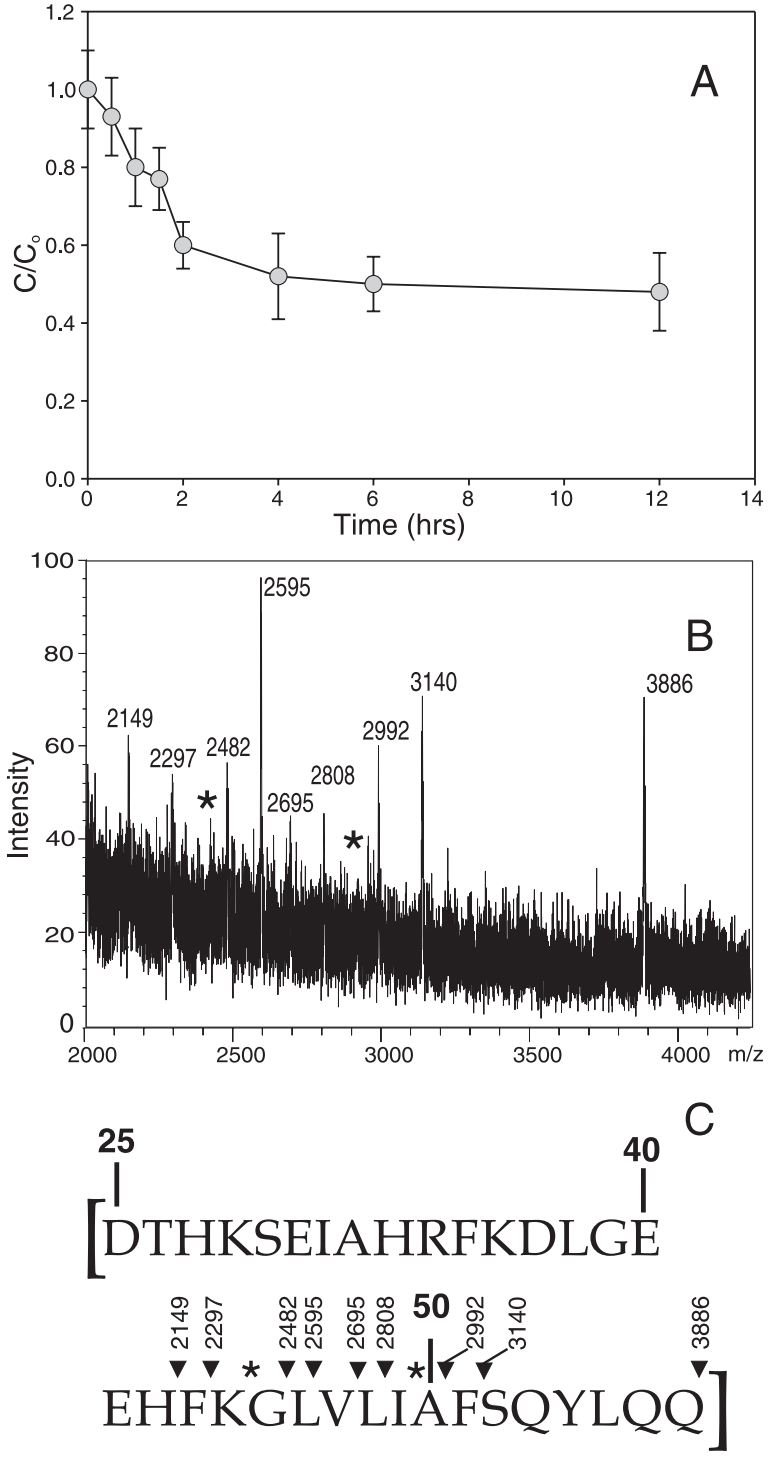
Bold numbers indicate peptides with one missed cleavage site.
[a] Amino acid position according to sequence data from http://ca.expasy.org/cgi-bin/peptide-mass.pl.

ments were observed throughout the initial degradation of the BSA (Fig. 2B). Knowing that the peptides were most likely from the added BSA, the search tool FindPept was used to identify components of the BSA molecule corresponding to the peptides observed in the MALDI-TOF-MS analysis. All peptide ions occurred at <4500 m/z and were components near the N or C terminus of the BSA molecule (Fig. 2C). One particularly well-represented peptide component from the parent BSA at 3886 Da (Glu25–Asp57) formed a series of peptides related by the sequential loss of one amino acid from the C terminus of the peptide (Fig. 2C).

3.4. Sedimentary degradation of BSA

The colorimetric CB method provided different results for the sediment pore water experiment relative to those obtained using the fluorescent CBQCA (Fig. 3B and D). CB measurements of protein (protein$_{CB}$) produced smoother, first-order losses with time (Fig. 3B). The fraction of BSA on the sediments increased primarily as a result of adsorption of the protein$_{CB}$ onto sediment particles (Fig. 3A). The fraction of the initial BSA in the pore water rapidly decreased as a result of both degradation of protein$_{CB}$ by bacteria and removal of protein$_{CB}$ from the dissolved phase onto the solid phase by adsorption (Fig. 3B). Using Fig. 3A to estimate adsorption, the first-order decay constant for degradation of protein$_{CB}$ alone could be calculated as 0.0395 h^{-1}. During the same experiment, protein measurements made on the solid and dissolved phase using CBQCA (protein$_{CBQCA}$) did not readily fit a decay function to determine the rate of removal (Fig. 3C and D). Measurements of the fraction of protein$_{CBQCA}$ on both the liquid and solid phases were erratic, sometimes exceeding the initial protein added ($C/C_o = 1.0$). For example, adding the fraction of protein$_{CBQCA}$ at 20 h from both the pore water and sedimentary phases yielded a C/C_o of 2.3. This demonstrates that the CBQCA measurement is sensitive to more than just whole proteins.

MALDI-TOF-MS analysis was carried out on pore water subsamples from the experiment to determine if peptide intermediates were created during the degradation of BSA (Fig. 4). The $[BSA+H]^+$ ion was visible, but because its signal compromised all other ions, >60,000-Da ions were deflected during the collection of data on smaller peptide intermediates. Signal-to-noise ratios of identified ions ranged from 1.6 to 11.7. Interpreting the m/z ratios as average masses of singly charged species, all ions could be assigned to the bovine serum albumin chain (Asp25–Ala607). Peptide fragments were 110–508 amino acids long. There are 583 amino acids in BSA (24 amino acids removed from the original 607 by Sigma-Aldrich during manufacturing of BSA), 582 potential cleavage sites, and 583! (factorial: $583 \times 582 \times 581 \ldots 3 \times 2 \times 1$) potential peptides. We used FindPept software to identify cleavage sites within the BSA sequence that generated continuous amino acid sequences bearing the molecular weights of peptides we observed ($<0.03\%$ tolerance). Mapping these peptides on the whole BSA sequence illustrates that the protein is primarily being cleaved from the C and N termini (Fig. 4B). The peptides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C/C$_o$
Time (hrs)
B
Intensity
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2297
2482
2595
2695
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m/z
C
25
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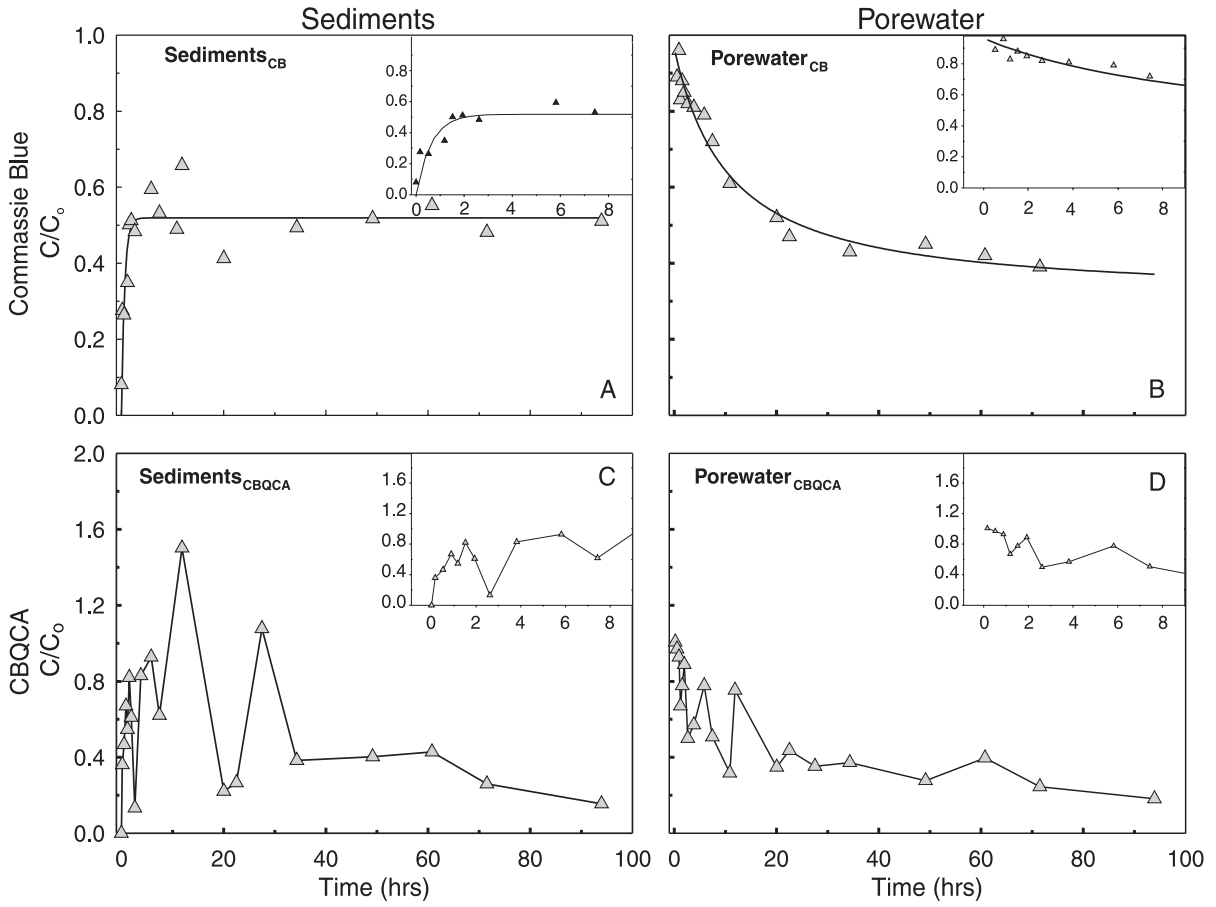
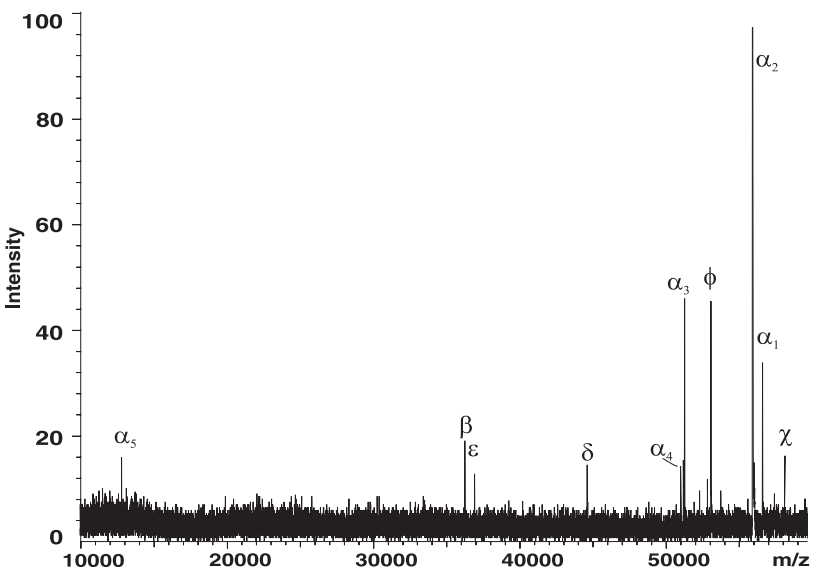


Fig. 3. Protein concentration at time t (C) relative to initial concentration (C_o) of protein added to the pore water versus time in decimal hours. Insets are close-ups of initial stages of degradation within the first 8 h, where sampling was more frequent. (A) Protein concentration from solid phase extractions measured by CB. (B) Protein concentration in pore water measured by CB. (C) Protein concentration in sediment extractions measured by CBQCA. (D) Protein concentration in pore water measured by CBQCA. Note different C/C_o scales between CB and CBQCA panels.

marked with an α illustrate how five peptides observed during the degradation with different N termini (Val78, Gly85, Phe126, Ser128, Thr462) can be reconstructed with a single C terminus (Glu572). The α_2 label (Gly85–Glu572) on the map marks the N terminus cleavage site necessary to reproduce the molecular mass of the dominant ion peak in Fig. 4A. By analyzing the cleavage specificity required to produce peptides with these masses, it is apparent that cleavage does not occur before or after one particular amino acid or 'group' of amino acids with similar functional groups.

Fig. 2. (A) Concentration of protein, as measured by CB, in whole seawater at time t (C) relative to initial concentration (C_o) of protein added versus time. (B) MALDI-TOF-MS spectra of the 2000–4200 m/z range of BSA fragments in whole seawater at the 12-h time point. (C) Peptide sequence for the 3886 m/z peptide from panel (B). The arrows denote where the cleavages are to produce each of the other peptides in panel (B). The asterisks (*) in both panels (B) and (C) indicate cleavages either missed by an endoprotease or not observed in the MALDI-TOF-MS analysis.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Intensity
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40000
50000
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α5
β
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α3
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α1
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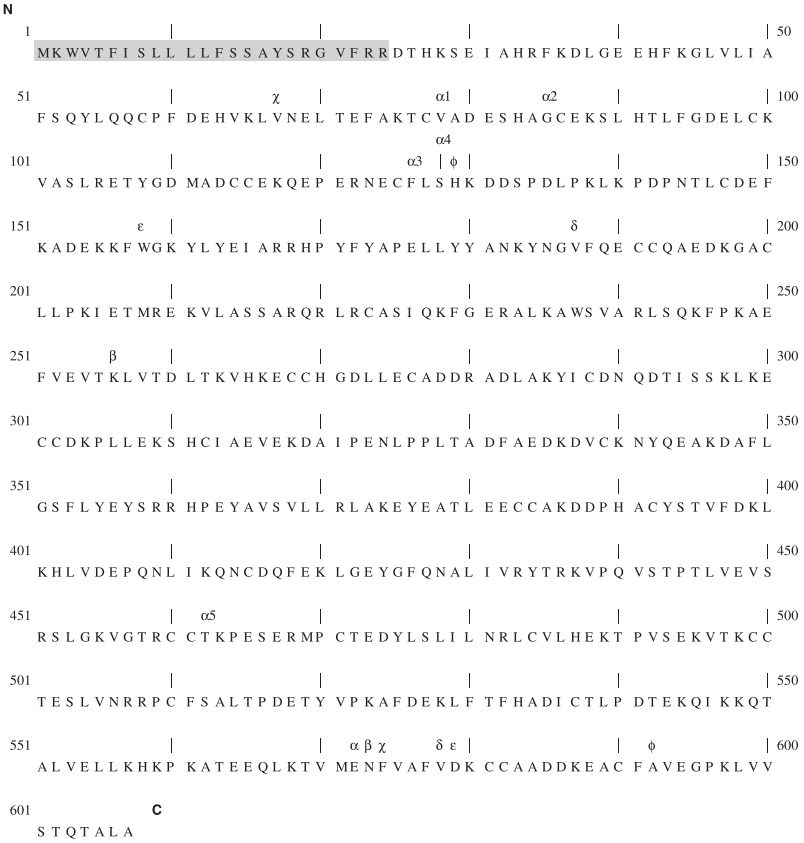
B
N
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FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
C
STQTALA

4. Discussion

Using a Bruker Bioflex III MALDI-TOF-MS, we are able to resolve the molecular weights of proteins in saltwater, without purification, in concentrations as low as 10 nM BSA. This corresponds to roughly 0.3 mg BSA carbon l^{-1}. Given that oceanic surface waters are roughly 1 mg carbon l^{-1} and that proteins are likely to be only a small component of that, the Bruker Bioflex III MALDI-TOF-MS system is nearing the capability of identifying BSA-sized proteins in seawater at ambient concentrations. Future improvements will place such direct protein measurements within our grasp. In the meantime, concentration of seawater DOM using chromatographic or ultrafiltration steps may allow for analysis of proteins in seawater using MALDI-TOF-MS (Doucette et al., 2000; Tanoue, 1996). Proteins and peptides could thus be analyzed intact, without hydrolysis to component amino acids. This would greatly facilitate determining the sources and sequences of the 'combined amino acids' found in the marine environment. Numerous aspects of the MALDI-TOF-MS method can be fine-tuned to improve results (Gobom et al., 1999; Kussmann et al., 1998; Mirgorodskaya et al., 2000).

To use MALDI-TOF-MS for the analysis of peptides and proteins in seawater, it was first necessary to confirm that the complex salt matrix did not significantly alter the ability of proteomic software packages (such as PeptMass, PeptIdent, etc.) to identify known peptides. We compared the actual m/z of peptides produced by a tryptic digestion of BSA in seawater (1.5 μM) to those predicted by the software PeptMass. The twenty-two peptides produced after 5 hours of digestion were all positively identified and matched predicted peptides from the original protein with no missed cleavage sites (Table 1). Then, assuming nothing about source protein (BSA) but acknowledging that the digest was conducted using trypsin, the same digest peptides were analyzed using PeptIdent (http://ca.expasy.org/tools/peptident.html) to scan for matches to any known protein. The molecular weights for these peptide fragments were cleanly resolved and can account for 37.4% of the sequence for BSA (score of 88%; Table 2). All other matches for our data were for other albumins, which had considerably lower scores. Other investigations, such as Doucette et al. (2000), have resolved 21 BSA peptide fragments from a tryptic digestion of 500 nM in deionized water, covering 34.6% of the BSA sequence. Thus, even though the digestion was conducted within seawater, the peptides generated were still conducive to analysis using established protocols, and when the peptides were submitted as 'unknowns,' the match to the original protein was excellent.

Table 2
Protein matches identified by PeptIdent for a trypsin digest of an unknown mammalian protein

Score	Number of peptide matches	% Sequence covered	Protein name
0.88	21	37.4	bovine serum albumin
0.42	10	14.8	sheep serum albumin
0.38	9	13.4	dog serum albumin
0.33	9	12.5	mouse hexosaminidase B
0.29	8	11.1	cat serum albumin
0.29	7	9.3	rat acyl-CoA
0.25	7	7.5	rat fructose-2,6-bisphosphatase
0.21	6	6.2	*Rhesus macaque* serum albumin
0.21	5	9.6	rabbit serum albumin
0.21	5	6.9	rat serum albumin

To successfully examine hydrolysis preferences by natural bacteria, seawater degradation experiments were conducted with 1 μM BSA solution. This is an unnaturally high concentration and is the unavoidable consequence of our desire to investigate peptides generated during the experiment without any sample preparation other than spotting ~ 1 μl on the MALDI-TOF-MS target. Peptides were generated at all time points, but were best resolved in the time point at 12

Fig. 4. (A) MALDI-TOF-MS spectrum from pore water collected after 1 h during bacterial degradation of BSA in Puget Sound sediments. Spectrum was obtained from a dried droplet of 0.7 μl pore water with 0.7 μl DHB matrix. (B) Amino acid sequence for the whole BSA precursor. The first 24 amino acids are removed by Sigma-Aldrich during the processing procedures for BSA (shaded portion of sequence). Peptides with molecular weights equivalent to the singly charged ions seen in the MALDI spectrum are denoted with Greek letters. The top left hand corner is the N terminus (site 1), and the last amino acid represents the C terminus (site 607). The α-series of peptides all have the same C terminus, but different N termini. Molecular weights of the peptides are α_1: 56,570, α_2: 55,890, α_3: 51,250, α_4: 50,950, α_5: 12,760, β: 36,240, χ: 58,080, δ: 44,590, ε: 36,900, and ϕ: 53,050.

h (Fig. 2). All the fragments observed were roughly 40 amino acids in length (<4500 Da). In many cases, the peptides were related by the sequential loss of one amino acid, indicative of the action of an exoprotease. Other than this, there was no pattern in the observed peptides to suggest preferential digestion by a specific type of protease (e.g., trypsin, etc.). Despite extensive searches using different matrices and MALDI-TOF-MS settings, we did not observe the presence of any larger peptides that might correspond to the middle of the protein. This suggests that perhaps the BSA molecule was associated with the surfaces of bacterial cells and that the peptides observed in the water were cleaved and released by cell-surface-associated proteases (Pantoja and Lee, 1994).

Degradation of BSA in a sedimentary system yielded slightly different results. Bulk concentrations of proteins were measured by two methods, Coomassie Blue dye and the CBQCA fluorescent tag. Although each assay was performed on the same subsamples, the CB graphs illustrate model-like degradation and adsorption, while the CBQCA graphs display abrupt protein enrichments and depletions through time (Fig. 3). We conducted a second, sterile experiment to determine the applicability of the CBQCA assay in sedimentary systems. BSA was slurried with UV-oxidized seawater and dry, organic-free chlorite (data not shown). Both the CBQCA and CB assays yielded similar concentrations of BSA through time and recorded only the effects of adsorption. Both methods measure equivalent bulk protein concentrations if the standard is whole and structurally and chemically similar to the measured protein. This indicates that perhaps the CBQCA data from our initial experiment with live microorganisms is valid. Thus, while the details of applying CBQCA to marine systems remain to be further worked out, the data we have obtained to date suggests that there may be valid information contained within the discrepancy between the two assays we used. Theoretically, discrepancies could arise between the CB and CBQCA assays when the BSA is cleaved to produce multiple peptides. CB dyes specific residues and is limited to proteins greater than 3000–5000 Da (Bradford, 1976; Mayer et al., 1986); thus, a protein concentration derived from this assay will remain constant whether the protein is whole or fragmented into multiple peptides. However, the CBQCA assay targets primary amines; thus, production of multiple peptides from a single protein will generate multiple primary amines and hence, a higher CBQCA signal. In our experiment, BSA was clearly hydrolyzed to smaller components (validated with our MALDI-TOF-MS data), and we hypothesize that in our experiment, CB measures the bulk protein signature, while CBQCA records the pulsed production and depletion of peptides produced from the degradation of BSA. This would imply that pulses of increased protein concentration, as measured by CBQCA, represent hydrolysis events and the creation of primary amines. Depletion of $\text{protein}_{\text{CBQCA}}$ results from the mineralization of the previously created peptides. Once the number of primary amines is depleted in the system, another hydrolysis pulse is initiated, creating more peptides for uptake. The increased concentration of peptides, in turn, activates the release of proteases into pore water to hydrolyze the peptides for cellular uptake. Our data are also consistent with the idea that at least some peptide intermediates are released back into the pore water in sedimentary systems, rather than diffusing directly to the cell membrane for utilization. Observations of peptide hydrolysis in seawater and sedimentary incubations led Pantoja et al. (1997) to suggest that this process is catalyzed by ecto- or extracellular enzymes. The model of extracellular enzyme foraging by Vetter et al. (1998) supports this theory. The model proposes that extracellular enzymes from sedimentary-attached bacteria solubilize substrates in excess of their growth requirements. This is counter-intuitive to the foundation of the optimal foraging theory, but the MALDI-TOF-MS analysis of the sedimentary degradation experiment also supports this model with evidence of BSA fragments present in pore water (Fig. 4A).

Analysis of the molecular mass fingerprint from the degradation of a known protein allowed us to determine trends during natural bacterial cleavage (Fig. 4B). Mapping all the observed potential peptides illustrates that the BSA molecule is more frequently cleaved at the N terminus. In numerous cases, the cleavage site at the C terminus was held constant, while cleavages at different amino acids on the N terminus were made to create a variety of peptides (e.g., α of Fig. 4B). Greater confidence lies in the identification of the amino acid sequence for

larger peptides because there are fewer possible permutations that can yield molecular weights within 0.03 % tolerance. The high proportion of these larger molecular weight peptides (55,890, 56,570, 53,050 and 51,250) provides evidence of the type of enzymes involved in initial stages of sedimentary degradation. Long strings of the original intact amino acid sequence suggest that amino acids have been clipped from the ends of the protein. Exopeptidases, which can sequentially remove amino acids from either the N terminus (aminopeptidase) or C terminus (carboxypeptidase) of proteins, perform this type of cleavage. Endopeptidases, on the other hand, such as trypsin, typically cleave the protein backbone adjacent to amino acids with specific functional groups. It appears that in the sediments, protein hydrolysis is predominantly catalyzed by nonspecific N terminus exopeptidases.

Analysis of the peptide maps from both the seawater and sediment incubations also suggests that hydrolysis can be sequential. It has been previously suggested that hydrolysis is not sequential, but rather, nonspecific enzymes preferentially cleave the second peptide bond from the C terminus during a sedimentary incubation of small polypeptides (Pantoja and Lee, 1999). An alternative explanation of their observations may involve the effects of the fluorescent tag on degradation. Although they demonstrated that the fluorescent lucifer yellow (LYA) tag had no observable effect on bacterial degradation, the tag was attached to the N terminus of the short chain polypeptides (two to eight amino acids), and they note that this may have hindered other potential cleavages by available enzymes. Mapping the peptides observed in the pore water and seawater degradations of this study (Figs. 2c and 4b) illustrates that there was no site-specific, or amino acid-specific, cleavage. This may either be the result of a nonspecific protease or mixture of bacterial proteases.

Both seawater and sedimentary bacterial degradation of BSA have confirmed that peptide intermediates are created and released back into the medium. To our knowledge, this is the first time cleavage site details have been observed in the hydrolysis of an intact protein into peptide fragments. Rather than the peptide intermediates being directly shuttled to the bacteria, some appear to be liberated and available for further enzymatic attack. Assuming complete degradation of the protein occurs, peptide intermediates could be transferred between bacterial groups. Excess solubilized substrate might then be consumed by passive assimilators, thereby achieving a more optimal foraging model for particle-attached bacterial extracellular enzymes (Vetter, 1998). By studying DOM uptake by different phylogenetic groups of bacteria in seawater, Cottrell and Kirchman (2000) revealed that a minimum of three bacterial components must be considered to correctly model the transfer of high-molecular-weight DOM to low-molecular-weight DOM. Cottrell and Kirchman (2000) focused on seawater uptake of DOM, so this generalization may not wholly apply to sedimentary environments. One of the most striking differences between the seawater and sedimentary degradation of proteins was the resulting size of the peptide intermediates created. Sedimentary digestions of BSA produced fragments that are a factor of 10 larger than those generated in the seawater. By monitoring two different bacterial isolates during the degradation of free ^{3}H-BSA and adsorbed ^{3}H-BSA, Nagata and Kirchman (1996) observed that both strains degrade free ^{3}H-BSA at the same rate, while adsorbed ^{3}H-BSA was not detectably degraded by one strain at all. Their evidence, in combination with the presented results, may indicate the presence of different phylogenetic groups of bacteria in sediments with different metabolic capacities. The sedimentary bacteria may have adopted this behavior as a response to their typical available food source. Aguilar et al. (1998) demonstrated that when trypsin was applied to proteins attached to reverse-phase chromatographic sorbent, specific regions of the protein remained intact. These specific regions were discovered to correspond to the hydrophobic domains of the protein. This clearly demonstrated that the hydrophobic domains of the protein were protected as a result of being bound to the support. The hydrophilic domains, on the other hand, were exposed to the solvent, available for enzymatic attack. If the majority of protein in a sedimentary system is particle bound, then it is likely that bacteria have developed enzymes that cleave off exposed amino acids (e.g., the N or C terminus of BSA).

The monitoring of peptide creation and hydrolysis patterns in both sedimentary and pelagic environments can provide the basis of a model for between-

species trophic-transfer of high-molecular-weight DOM to its low-molecular-weight counterparts. Analysis of the intact proteins present in both sedimentary and pelagic environments will better help us understand by what means bacteria control the particulate and dissolved DOM pool. Analytical techniques, such as MALDI-TOF-MS, are becoming more tolerant to the harsh analytical conditions present in oceanic environments, including low analyte concentrations in combination with high levels of salts and other contaminants. More studies must be conducted to monitor the transfer of particulate proteins and peptides as they journey from pelagic to benthic bacterial communities. Understanding some of these initial steps in bacterial degradation of protein and peptides will help decipher the circulation of proteinacous material in sediments and determine precursors for long-term preservation of both DOC and DON.

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