Hydrolysis of peptides in seawater and sediment

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Abstract

Protein hydrolysis and subsequent peptide production appear to be the links between degradation of protein and production of free amino acids in the marine environment. This model has not yet been fully demonstrated because neither presence of peptides nor peptide hydrolysis has been directly measured in seawater or sediments.

Fluorescent Lucifer Yellow (LYA)-derivatives of several peptides were synthesized and tested for use as models in an investigation of peptide hydrolysis in seawater and sediment. We demonstrated that these LYA-derivatives behave to some degree as the natural peptides by showing that LYA-dialanine effectively competes with dialanine (ala₂) for the active sites of microbial hydrolytic enzymes found in seawater. LYA-derivatives of ala₁, ala-leu and ala₃ hydrolyzed to smaller peptides or free amino acids in both seawater and sediments. In seawater, hydrolysis of the longer peptide, LYA-ala₄ was 90 X faster than hydrolysis of LYA-ala₂ and 30 X faster than LYA-ala-leu. In sediments, rapid disappearance of the initially-added substrate LYA-ala₄ from pure waters was followed by slower production of LYA-ala₂, LYA-ala₃ and LYA-ala₁. Hydrolysis was not random; preferential cleavage of certain peptide bonds occurred.

Keywords: extracellular hydrolysis; peptides; amino acids; fluorescent peptide analogs; sediment diagenesis; organic matter decomposition

1. Introduction

Amino acids, peptides and proteins are a major reservoir of organic nitrogen in the marine environment. Nitrogen in amino acids from acid-hydrolyzable proteinaceous material accounts for ~ 50% of PON in the water column (Tanoue, 1992), and 40-50% of sinking particulate organic nitrogen (C. Lee and Cronin, 1982; Ittekkot et al., 1984). Among the degradative processes occurring on particles, hydrolysis of proteins and peptides seems to be predominant (Smith et al., 1992). Due to decomposition, only a small portion of the amino acids produced in the euphotic zone reaches surface sediment (C. Lee and Cronin, 1982; Wefer et al., 1982; Ittekkot et al., 1984), where proteinaceous material makes up 30-40% of the organic nitrogen (Henrichs et al., 1984; Burdige and Martens, 1988).

Decomposition of proteins and peptides can occur inside or outside a cell. In the water column, much of the phytoplankton protein produced is consumed by animal grazers. Ingestion of particulate detritus by benthic animals also degrades protein (J.J. Lee, 1980; Plante et al., 1990). For bacteria to decompose protein, hydrolysis must occur outside the cell, since bacteria can only transport relatively small substrates (MW < ~ 600 daltons) across their cell membranes (Alexander, 1973; Payne, 1980). This hydrolysis can be due to free (extracellular) enzymes or to attached ectoenzymes. Breakdown of protein macromolecules
produces small peptides and amino acids capable of being further degraded or used in protein resynthesis.

Proteins and peptides reaching surface sediments are further decomposed at the sediment-water interface and deeper in the sediments (Whelan, 1977; Maita et al., 1982; Henrichs et al., 1984). In sediments, these compounds are adsorbed onto particles which may slow their decomposition (Sugai and Henrichs, 1992; Mayer, 1994; Luo and Henrichs, 1997a).

Protein hydrolysis is difficult to measure in the marine environment. Although several studies have measured combined hydrolysis and uptake of radiolabeled protein (e.g., Hollibaugh and Azam, 1983; Keil and Kirchman, 1992; Taylor, 1995), “peptide-like” fluorogenic substrates are more commonly used to determine general peptide bond hydrolysis rates (Hoppe, 1983; Somville and Billen, 1983; Hashimoto et al., 1985). For example, aminopeptidase (N-terminal) cleavage of the amide bond in leucine-methylcoumarinylamide (Leu-MCA) yields a fluorescent product (Kanaoka et al., 1977) and has been used to measure hydrolysis rates in seawater and sediments (Rheinheimer et al., 1989; Boetius and Lochte, 1994). A different approach to the study of peptide decomposition was recently developed by Luo and Henrichs (1997b). They elongated the C-terminal end of several amino acids and oligopeptides with a tritiated amino acid. Hydrolysis and decomposition of the resulting 3H-labeled peptides were then measured in anoxic marine sediments.

We report here a novel approach to studying the effects of structure and size on peptide hydrolysis rates in natural environments. We synthesized fluorescent molecular analogs of dialanine (ala2), trialanine (ala3), tetraalanine (ala4), alanyl-leucine (ala-leu), leucyl-alanine (leu-ala), leucyl-leucine (leu-leu), leucine (leu) and alanine (ala) and demonstrate here their use in measuring hydrolysis rates in seawater and sediment. Chemical and biochemical behavior of the analogs are compared to that of their natural counterparts. We also compare hydrolysis rates of the peptides in seawater with those obtained using Leu-MCA. Kinetic measurements using the fluorescent peptides are used to estimate rates of hydrolysis and natural concentrations of peptides in seawater. In sediments, the effects of both hydrolysis and adsorption of LYA-analogs are evaluated.

2. Experimental approach

2.1. Synthesis, structural characterization, and quantification of the fluorescent probes

Fluorescent derivatives of ala2, ala3, ala4, leu, ala-leu, leu-ala, leu-leu and leu were synthesized following the amidization procedure of Stewart (1981). Briefly, the N-terminal amine of a peptide (or amino acid) was condensed with Lucifer Yellow Anhydride (4-amino-3,6-disulfo-1,8-naphthalic anhydride; hereafter LYA) in refluxing aqueous acetate buffer at pH 5 at 105°C. LYA was obtained from Aldrich, amino acids and peptides from Sigma.

Reaction progress was monitored by high performance liquid chromatography using a Shimadzu 10AS HPLC with a Kratos UV absorbance detector at 272 nm. A 5-μm Beckman C-18 Ultrasphere-ODS column (25 cm × 4.6 mm) was used with a mobile phase of 0.05 M KH2PO4 (pH 4.5) and methanol (1 ml min-1). A gradient of 0–25% methanol in 25 min, then 25–50% in 5 min was used. Final reaction products were purified using reverse-phase (C-18) column chromatography with a mobile phase of water, then water–methanol and methanol, until the yellow band showing the fluorescent derivatives eluted. When necessary, the derivatives were purified by reverse-phase HPLC with 0.1% TFA–water and 0.1% TFA–acetonitrile, ramping the organic solvent to 25% in 20 min at 0.8 ml min-1. Fractions were reanalyzed by HPLC and showed a single UV-absorbance peak in each case.

1H-nmr spectra were obtained using Bruker AC-250 or AM-300 spectrometers at 250 and 300 MHz, respectively. The samples were dissolved in a little deuterium oxide and the solvent evaporated in vacuo to remove exchangeable protons. The sample was then dissolved in fresh D2O for spectral determination. The residual protons in the solvent were used as the internal standard (δ = 4.80 ppm).

The LYA-derivative of 3H-alanine was also prepared by adding 1 mCi of L-[3-3H]alanine to the reaction mixture containing LYA and alanine. Purification was as described earlier; HPLC properties were compared with authentic LYA-alanine whose structure had been previously confirmed by 1H-nmr.

LYA-derivatives containing alanine were quantified by HPLC using the KH2PO4 and methanol
mobile phase described above. Simultaneous separation of derivatives of leu-leu, leu-ala, ala-leu, ala-ala, ala and leu was achieved within 20 min using an initial 15-min gradient of 0–35% methanol. A fluorescence detector (Shimadzu RF-551) with excitation and emission wavelengths of 424 and 550 nm, respectively, was used to detect the probes. Response factors were compared with those of standards prepared in filtered seawater.

2.2. Evaluation of the fluorescent probes as peptide analogs

LYA-derivatives hydrolyze to form a fluorescent and a non-fluorescent product: a fluorescent amino acid or peptide probe still derivatized at the N-terminus, and an underivatized amino acid or peptide. Hydrolysis rates are measured by recording loss and appearance of the fluorescent substrate and products. Certain behavior differences between the fluorescent substrates and natural compounds were expected, and experiments were devised to quantify these differences. We carried out competition experiments between dialanine and its fluorescent derivative to compare enzymatic hydrolysis rates of the two compounds. If two substrates are sufficiently similar, they compete for the active site(s) of a microbial enzyme(s). If they compete, then addition of competitor (the natural peptide in this experiment) will result in an apparent increase in the half-saturation constant ($K_s$) of the fluorescent substrate. More substrate is needed to reach half of the maximum velocity, since the enzymes are hydrolyzing two similar substrates at the same time. At high concentrations of substrate, the system becomes saturated, and the reaction rate ($v$) reaches a constant maximum value ($V_m$) where the influence of the competitor is masked (Dixon and Webb, 1964).

In a competition experiment (8/8/94), Flax Pond (Stony Brook, New York, U.S.A.) seawater was incubated at 22–25°C with concentrations of LYA-ala$_2$, ranging from 0.03 to 2.5 μM. Ala$_2$ (1 μM) was added as the competitor to half of the incubation flasks. Rates of hydrolysis ($v$) of the probe were calculated for each concentration of fluorescent substrate with and without the competitor added. Using a Lineweaver–Burk plot, the reciprocal of the rate of hydrolysis ($1/v$) was plotted vs. reciprocal concentration of the substrate ($1/S$) to estimate the values of the kinetic parameters $V_m$ and $K_s$. The slope in such a plot corresponds to $K_s/V_m$ and the y-intercept is $1/V_m$.

To further quantify the effect of competition we compared the rate of LYA-ala$_2$ hydrolysis with that of an equimolar mixture of probe and natural peptide. LYA-ala$_2$ hydrolysis rates were measured in Flax Pond seawater (2/25/96) in two experiments. In one series of 15-ml flasks, the substrate was the peptide probe alone (38, 54, 107, 217, 520, 1917 and 3600 nM), and in the other the substrate was an equimolar mixture of LYA-ala$_2$ and ala$_2$ at the same total concentrations. Flasks were incubated in the dark and subsampled at various times. LYA-ala$_2$ hydrolysis rates were calculated from the disappearance of the probe as described later.

To determine how addition of the fluorescent tag affects adsorption behavior of the peptide, partition coefficients of $^{14}$C-alanine, IYA-ala$_2$, IYA-$^3$H-alanine, LYA-ala$_2$, and LYA-ala$_4$ were compared. Sediment slurries were prepared by adding 10 ml of filtered seawater to centrifuge tubes with weighed amounts (~3 g) of wet sediment from Flax Pond (11/8/96). Each compound was added to four tubes to obtain final concentrations of 0.25, 0.5, 0.75 and 1 μM. For alanine, a mixture of alanine and $^14$C labeled alanine (L-alanine-$UL$-$^{14}$C, 114 μCi μmol$^{-1}$) were added to achieve the desired concentrations. Samples were incubated at 0–1°C with continuous shaking for 2 h, then centrifuged (3600 rpm, 20 min). One ml of the supernatant from radioactive samples was mixed with 3 ml of Optifluor (Packard) and counted in a Packard Tri-Carb 1600CA scintillation counter for 5 min. Counts were corrected for quench using the transformed spectral index of the external standard spectrum with automatic efficiency control (tSIE/AEC). The amount of label adsorbed was calculated by subtracting the amount of label remaining in solution from the label initially added. We assume that $^{14}$C-alanine and $^3$H-alanine distribute between phases in the same manner as alanine. Where linear adsorption occurs, the slope of the relationship between moles adsorbed per g solid and moles remaining in solution per ml pore water is the partition coefficient, $K_{ads}$, in units of ml gdw$^{-1}$ (Wang and Lee, 1993). Samples containing
fluorescent probes were filtered after centrifuging and analyzed by HPLC as described earlier. $K_{\text{ads}}$ was calculated for each probe as described above.

To demonstrate that alanine was not mineralized in the autoclaved sediment during the adsorption experiments, production of $^{14}\text{CO}_2$ from $^{14}\text{C}$-alanine was measured. A 2-ml aliquot of the supernatant from the adsorption experiment described above was shaked with 40 $\mu$l of concentrated $\text{H}_2\text{SO}_4$ for 2 h to release $\text{CO}_2$. $^{14}\text{CO}_2$ was trapped on a paper wick soaked with 300 $\mu$l of Carbo-Sorb E (Packard) that was suspended above the acidified solution. The wick was placed in a scintillation vial containing 0.1 ml of $\text{HCl}$ (1 N), 0.1 ml of Tris buffer (pH 7) and 9 ml of Optifluor. Less than 3% of the initially added $^{14}\text{C}$-ala was recovered as $^{14}\text{CO}_2$. In the LYA-ala$_4$ experiment, production of LYA-ala$_2$ (30–66%) was detected after 2 h; both concentrations were pooled to calculate $K_{\text{ads}}$.

2.3. Peptide hydrolysis in seawater

Peptide hydrolysis was measured in unfiltered surface seawater samples taken from Flax Pond on 8/8/94, 5/7/95 and 6/24/96. Samples were amended with fluorescent probe to a final concentration of 50–100 nM, depending on the experiment. After various incubation times, subsamples of $\sim 2$ ml were filtered (0.2 $\mu$m Nuclepore) and frozen until analysis by HPLC (typical injection volume: 100 $\mu$l). Hydrolysis in the same samples was also measured with Leucine-methyl-coumarinylamide (Leu-MCA) according to the procedure of Hoppe et al. (1988). This technique is based on measuring an increase in fluorescence when the Leu-MCA substrate is hydrolyzed. The effects of abiotic hydrolysis and adsorption of the probes to suspended particles or to glassware was evaluated by measuring hydrolysis rates in seawater to which $\text{HgCl}_2$ (final concentration 40 mg l$^{-1}$) was added 30 min before addition of the substrate.

Initial first-order rate constants ($k$) of dipeptide hydrolysis were calculated from disappearance of substrate or production of product when $\leq 10\%$ of the reaction had occurred (Pantoja and Lee, 1994). When LYA-ala$_4$ was used as the substrate, its disappearance was used for the calculation, since its hydrolysis produces multiple fluorescent products. Production of MCA was used to calculate $k$ when Leu-MCA was the substrate of hydrolysis. In the experiment with LYA-ala$_4$ as the substrate, the reaction was followed until all peptides were hydrolyzed.

2.4. Kinetic parameters of enzymatic LYA-ala$_2$ and LYA-ala$_4$ hydrolysis in seawater

Seawater from Flax Pond (8/8/94 and 5/7/95) was incubated in 15-ml polypropylene flasks with either LYA-ala$_2$ (0.03–2.5 $\mu$M) or LYA-ala$_4$ (0.05–1.5 $\mu$M). Subsamples were removed over time and reactants and products analyzed by HPLC. Hydrolysis rate constants for each treatment at various concentrations of added substrate were calculated from the disappearance of substrate as described earlier. Hydrolysis rate was calculated as the product of rate constant times concentration of the added substrate. Values of $K_s$ and $V_m$ were estimated from linear fits to a Lineweaver–Burk plot as described above.

2.5. Hydrolysis and adsorption of peptides in sediment

Adsorption plus hydrolysis of LYA ala$_4$ was measured by following changes in pore water concentration of added fluorescent precursor and its hydrolysis products over time. Flax Pond sediment (3/29/95) from the surface 8 cm was sieved (1-mm screen; no water added) and homogenized; subsamples of $\sim 2$ g were placed in 15-ml centrifuge tubes. Then, 10 ml of 100 nM LYA-ala$_4$ in 0.2-$\mu$m-filtered seawater were added to each tube. No attempt was made to keep the sediment anoxic. The tubes were shaken for 100 h; after various incubation times one or two tubes were removed, centrifuged, and the supernatant filtered (0.2 $\mu$m Nuclepore) and analyzed by HPLC. Porosity of the slurry and dry solid density of the sediment were also determined. Disappearance of LYA-peptides from solution and subsequent production of hydrolysis products were modeled as first order processes. A rate constant for hydrolysis plus adsorption of LYA-ala$_4$ was estimated by fitting the function $C = C_0 \exp(-kt)$ to initial time course data. Loss of LYA-ala$_2$ was similarly modeled to obtain a
Table 1

<table>
<thead>
<tr>
<th>Probe</th>
<th>MW (g/mol)</th>
<th>δ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYA-alal</td>
<td>463.1</td>
<td>1.65 (d, $J = 7.0$ Hz, 3H, CH$<em>3$) 5.46 (q, $J = 7.0$ Hz, 1H, CH), 8.72 (s, 1H$</em>{arom}$), 8.79 (s, 1H$<em>{arom}$), 8.93 (s, 1H$</em>{arom}$)</td>
</tr>
<tr>
<td>LYA-alal$_2$</td>
<td>534.2</td>
<td>1.27 (d, $J = 7.0$ Hz, 3H, CH$<em>3$), 1.73 (d, $J = 6.9$ Hz, 3H, CH$<em>3$), 4.25 (q, $J = 7.2$ Hz, 1H, CH), 5.72 (q, $J = 6.9$ Hz, 1H, CH), 8.66 (s, 1H$</em>{arom}$), 8.73 (s, 1H$</em>{arom}$), 8.79 (s, 1H$_{arom}$)</td>
</tr>
<tr>
<td>LYA-alal$_3$</td>
<td>698.8</td>
<td>1.31 (d, $J = 7.2$ Hz, 3H, CH$<em>3$), 1.43 (d, $J = 7.3$ Hz, 3H, CH$<em>3$), 1.65 (d, $J = 6.8$ Hz, 3H, CH$<em>3$), 4.14 (q, $J = 7.2$ Hz, 1H, CH), 4.39 (q, $J = 7.3$ Hz, 1H, CH), 5.74 (q, $J = 6.8$ Hz, 1H, CH), 8.72 (s, 1H$</em>{arom}$), 8.80 (s, 1H$</em>{arom}$), 8.95 (s, 1H$</em>{arom}$)</td>
</tr>
<tr>
<td>LYA-alal$_4$</td>
<td>770.8</td>
<td>1.04 (d, $J = 7.2$ Hz, 3H, CH$_3$), 1.40 (d, $J = 7.3$ Hz, 3H, CH$<em>3$), 1.54 (d, $J = 7.0$ Hz, 3H, CH$<em>3$), 1.73 (d, $J = 7.0$ Hz, 3H, CH$<em>3$), 3.90 (q, $J = 7.3$ Hz, 1H, CH), 4.38 (q, 2H, 2×CH), 5.71 (q, $J = 7.0$ Hz, 1H, CH), 8.68 (s, 1H$</em>{arom}$), 8.77 (s, 1H$</em>{arom}$), 8.99 (s, 1H$</em>{arom}$)</td>
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<tr>
<td>LYA-alal-leu</td>
<td>670.8</td>
<td>0.58 (d, $J = 6.5$ Hz, 3H, CH$_3$), 0.83 (d, $J = 6.4$ Hz, 3H, CH$<em>3$), 1.12 (m, 1H, CH), 1.43 (broad t, 2H, CH$<em>2$), 1.74 (d, $J = 6.9$ Hz, 3H, CH$<em>3$), 4.15 (t, $J = 7.5$ Hz, 1H, CH), 5.64 (q, $J = 6.9$ Hz, 1H, CH), 8.62 (s, 1H$</em>{arom}$), 8.70 (s, 1H$</em>{arom}$), 8.77 (s, 1H$</em>{arom}$)</td>
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<tr>
<td>LYA-leu-alal</td>
<td>576.31</td>
<td>0.90 (d, $J = 6.5$ Hz, 3H, CH$_3$), 0.97 (d, $J = 6.5$ Hz, 3H, CH$<em>3$), 1.31 (d, $J = 7.3$ Hz, 3H, CH$<em>3$), 2.07 (m, 1H, CH), 2.06 (m, 2H, CH$<em>2$), 4.4 (q, $J = 7.2$ Hz, 1H, CH), 5.8 (t, 1H, CH), 8.81 (s, 1H$</em>{arom}$), 8.88 (s, 1H$</em>{arom}$), 9.10 (s, 1H$</em>{arom}$)</td>
</tr>
<tr>
<td>LYA-leu-leu</td>
<td>714.81</td>
<td>0.57 (d, $J = 6.6$ Hz, 3H, CH$_3$), 0.79 (d, $J = 6.6$ Hz, 3H, CH$<em>3$), 0.89 (d, $J = 6.6$ Hz, 3H, CH$<em>3$), 0.96 (d, $J = 6.6$ Hz, 3H, CH$<em>3$), 1.18 (m, 1H, CHH'), 1.41 (m, 2H, 2×CH), 1.55 (m, 1H, CHH'), 1.92 (m, 1H, CHH'), 2.11 (m, 1H, CHH'), 4.20 (t, $J = 7.3$ Hz, 1H, CH), 5.69 (m, 1H, CH), 8.74 (s, 1H$</em>{arom}$), 8.82 (d, $J = 1.3$ Hz, 1H$</em>{arom}$), 9.04 (d, $J = 1.3$ Hz, 1H$</em>{arom}$)</td>
</tr>
<tr>
<td>LYA-leu-leu</td>
<td>505.10</td>
<td>0.89 (d, $J = 6.5$ Hz, 3H, CH$<em>3$), 0.97 (d, $J = 6.5$ Hz, 3H, CH$<em>3$), 1.52 (m, 1H, CH), 2.04 (m, 2H, CH$<em>2$), 5.80 (m, 1H, CH), 8.80 (s, 1H$</em>{arom}$), 8.87 (s, 1H$</em>{arom}$), 9.08 (s, 1H$</em>{arom}$)</td>
</tr>
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</table>

Chemical shifts (δ) are in ppm. s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet. Molecular weights were calculated for the Li salt of the compounds, except for LYA-leu-leu which was isolated as the K salt.
hydrolysis rate. Rate constants for the net production of LYA-alal, LYA-alal and LYA-ala were calculated by fitting the function $P = C_0 \exp(-kt) + C_o$ (where $P$ = hydrolysis product formed with time) to the experimental data that showed increase in concentration in pore water.

3. Results

3.1. Synthesis and chromatography of the fluorescent probes

The LYA-derivatives we synthesized are stable, water soluble, fluorescent imides. All derivatives
could be structurally characterized by high-resolution $^1$H-NMR spectroscopy since they gave simple first-order spectra which were readily analyzed (Table 1). All showed three distinct downfield singlets in the 8.6–8.8-ppm region due to the three aromatic protons of the fluorescent tag. The proton on the α-carbon of the LYA-derivatives was shifted significantly downfield (∼1.3 ppm) compared to the proton in the parent peptide. Integration of the spectra gave areas which corresponded to the expected number of protons.

All probes had fluorescence maxima at the same wavelengths (424-nm excitation, 550-nm emission), but exhibited different HPLC retention times as shown for LYA-peptides containing alanine (Fig. 1). The detection limit for each probe was 0.25 pmol or 0.5 nM for a 500-μL injection of seawater or pore water. Peak-to-noise-ratio was 1.9 for LYA-ala and 1.4 for LYA-ala₂. Variability of duplicate analysis (CV) of seawater was <1% at 20 nM concentrations, and in sediments 7% at 70 nM. Response factors (measured as nM area$^{-1}$) of the various probes differ by ±10%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{ab}$</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>alanine</td>
<td>45.8</td>
<td>3.38</td>
</tr>
</tbody>
</table>
| L YA-alanine         | ~0      | n.a.
| L YA-(^3)D alanine   | ~0      | n.a.
| L YA-allyl-alanine   | 0.3     | 0.03|
| L YA-tetraalanine    | 1.7     | 0.25|

Units are ml gdw$^{-1}$. SE is the standard error of the isotherm fitting. n.a. = not applicable.
3.2. Evaluation of the fluorescent probes as peptide analogs

In seawater, competitive inhibition of LYA-ala₂ hydrolysis was observed in the presence of dialanine. The half-saturation constant of hydrolysis ($K_s$) increased from $96 \pm 30 \text{ nM}$ (uninhibited) to $359 \pm 43 \text{ nM}$ (inhibited) after addition of ala₂. The maximum velocity, $V_m$, of hydrolysis remained unaltered, $0.3 \text{ nM h}^{-1}$ in both cases (Fig. 2A). Inhibition of LYA-ala₂ hydrolysis was also observed in the second competition experiment where equimolar amounts of natural peptide and probe were added (Fig. 2B). At hydrolysis rates below $V_m$, rates in the presence and absence of ala₂ were approximately equal. At higher substrate concentrations where hydrolysis rates equal $V_m$, the replacement of 50% of LYA-ala₂ by ala₂ decreased the hydrolysis rate of the probe to two-thirds of that observed when LYA-ala₂ was the only substrate.

In sediment slurries, partition coefficients of LYA-alanine and LYA-peptides were very low ($0–1.7 \text{ ml gdw}^{-1}$) compared to the partition coefficient of alanine, $45.8 \text{ ml gdw}^{-1}$ (Table 2). $K_{ads}$ increased with the number of amino acids in the peptide, with $K_{ads}$ of LYA-ala₂ > LYA-ala₃ > LYA-ala (Table 2).
Table 3
Hydrolysis rate constants in seawater of fluorescent alanine peptides and leucine-MCA, and LYA-dipeptides containing leucine and alanine

<table>
<thead>
<tr>
<th>Substrate</th>
<th>5/7/95</th>
<th>SD</th>
<th>6/24/96</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYA-ala₂</td>
<td>0.323</td>
<td>0.014</td>
<td>0.315</td>
<td>0.007</td>
</tr>
<tr>
<td>LYA-ala₃</td>
<td>0.004</td>
<td>0.0007</td>
<td>0.001</td>
<td>0.0002</td>
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<tr>
<td>LYA-ala-leu</td>
<td>0.011</td>
<td>0.0014</td>
<td>0.057</td>
<td>0.0099</td>
</tr>
<tr>
<td>Leu-MCA</td>
<td>0.039</td>
<td>0.0031</td>
<td>0.020</td>
<td>0.0007</td>
</tr>
<tr>
<td>LYA-leu-leu</td>
<td>0.002</td>
<td>0.0006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYA-leu-ala</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


d is the standard deviation of k from duplicate flasks. Substrates were added individually.

Luo and Henrichs (1997a) also found this effect; adsorption of tritium-labeled dialanine was generally similar or two times greater than alanine in Resurrection Bay and Skan Bay sediments, respectively.

3.3. Peptide hydrolysis in seawater

Addition of LYA-ala₂ (50 nM) to an unfiltered seawater sample taken from Plax Pond resulted in the loss of substrate with time and the appearance of a fluorescent hydrolysis product whose retention time coincides with that of authentic LYA-alanine (Fig. 3). In this experiment, the substrate completely disappeared after ~20 days. Hydrolysis rate constants of LYA-ala₂ were calculated from both disappearance (k = 0.0038 ± 0.0007 h⁻¹) of LYA-ala₂ from seawater and from production (k = 0.0016 ± 0.0004 h⁻¹) of LYA-alanine. These values are not significantly different within 95% confidence limits. Throughout the incubation, the sum of the concentrations of substrate plus product was nearly 100% of the added substrate. Mercuric chloride drastically slowed hydrolytic activity in seawater; only 2% of the substrate was hydrolyzed after 20 days of incubation.

Hydrolysis rate constants of the fluorescent derivatives in seawater were noticeably different from each other and from the hydrolysis of Leu-MCA (Table 3). Hydrolysis of LYA-ala₄ was 30 and 90 times faster than hydrolysis of LYA-ala-leu and LYA-ala₂, respectively. The hydrolysis rate of the fluorogenic substrate Leu-MCA was between that of LYA-ala₄ and LYA-ala-leu. Incubation of LYA-ala₄ in seawater was followed for 800 h, resulting in sequential hydrolysis of the substrate to smaller peptides and amino acids (Fig. 4). The main hydrolysis product was LYA-ala₂, which slowly hydrolyzed to form LYA-ala. Production of LYA-ala₂ was also detected at the beginning of the incubation, but only accounted for ~5% of the originally added substrate (Fig. 4).

3.4. Kinetic parameters of enzymatic hydrolysis of LYA-ala₂ and LYA-ala₄ in seawater

The half-saturation constant estimated for the hydrolysis of LYA-ala₂ was 487 ± 88 nm, much larger than the Kₛ value estimated for LYA-ala₂ in seawater (Table 4). The maximum rate of hydrolysis (Vₘ) of the tetrapeptide, was 1600 times larger than that of the dipeptide (333 vs. 0.2 nM h⁻¹, respectively). These values for the Michaelis–Menten parameters are in the range measured for peptide hydrolysis in seawater using the fluorogenic substrates leu-MCA (Rheinheimer et al., 1989) or leucyl-β-naphthylamide (Billen, 1991). Rate constants under substrate

Table 4
Kinetic parameters of LYA-ala₂ and LYA-ala₄ in Flax Pond seawater (8/8/94 and 5/7/95), and estimates of natural concentrations [Sₙ] and turnover times (τ) of ala₂ and ala₄

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kₛ (nM)</th>
<th>Vₘ (nM h⁻¹)</th>
<th>Vₘ/Kₛ (h⁻¹)</th>
<th>k (h⁻¹)</th>
<th>Kₛ+Sₙ (nM)</th>
<th>Sₙ (nM)</th>
<th>τ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYA-ala₂</td>
<td>95 ± 30</td>
<td>0.3</td>
<td>0.003</td>
<td>0.002</td>
<td>250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LYA-ala₄</td>
<td>487 ± 88</td>
<td>333</td>
<td>0.68</td>
<td>0.44</td>
<td>415</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ala₂</td>
<td>120–180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>731</td>
</tr>
<tr>
<td>ala₄</td>
<td>0–16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
</tbody>
</table>

Errors are 95% confidence limits of the mean. See text for definition of terms.
3.5. Loss of LYA-ala₄ in Flax Pond sediments by hydrolysis and adsorption

When added to Flax Pond sediment, LYA-ala₄ totally disappeared from pore water in 1.5 h (Fig. 5A). Production of LYA-ala₂ was detected at the first sampling time (2–10 min). The transient production of LYA-ala₃ was seen before it disappeared from solution during the first 6 h of incubation (Fig. 5B). LYA-ala was first detected in solution at ~1.5 h, mainly from the hydrolysis of LYA-dialanine (Fig. 5B). LYA-ala is stable to further hydrolytic attack and accounted for 100% of the fluorescence in the pore water fraction at the end of the incubation (Fig. 5C).

The rate constant for the disappearance of LYA-ala₄ from solution (hydrolysis plus adsorption) was 3.1 h⁻¹; rate constants for appearance of LYA-ala₃, LYA-ala₂, and LYA-ala were estimated as 0.05, 0.8 and 0.03 h⁻¹, respectively (Fig. 5). After LYA-ala₂ peaks (Fig. 5A), it disappears from solution with a rate constant of 0.043 h⁻¹, which corresponds to a turnover time of 23 h. Thus, LYA-ala₂ is turned over 72 times slower than LYA-ala₄, similar to the 80:1 ratio found when LYA-ala₄ and LYA-ala₂ were incubated separately (Table 3).

4. Discussion

4.1. Experimental approach

Fluorescent peptide derivatives allowed measurement of peptide hydrolysis rates at low substrate levels in both seawater and sediments. Peptide hydrolysis in these systems appears to be catalyzed by ecto- or extra-cellular enzymes, since all fluorescent substrates and their fluorescent products were present in seawater throughout the incubation (Fig. 3), and thus incorporation into cells was unlikely. The small peptides we studied (< 4 residues) would normally be transported across cell membranes (Nikaido and Vaara, 1985). However, the addition of 450 MW units from the LYA tag is a deterrent to this trans-
Peptide hydrolysis is biological in origin, since no hydrolysis was detected when the substrate was incubated in seawater previously spiked with HgCl₂, or in sediment that was autoclaved prior to incubation.

Even though LYA-peptides offer a simple, direct and rapid method for measuring hydrolysis rates in seawater and sediments, several potential biases exist. We evaluate these below. First, we compared the kinetic responses of natural microbial populations to both fluorescent and natural substrates (Fig. 2A). Results of the competition experiment between LYA-ala₂ and ala₂ suggested that peptide analogs can compete with undervatized dipeptides for the same enzyme active sites. We also quantified similarity of the two substrates (LYA-ala₂ and ala₂) by comparing hydrolysis rate of the probe alone with that of an equimolar mixture of probe and peptide. If the probe and peptide are competing equally with each other at rates equal to \( V_n \), the hydrolysis rate of the probe in the presence of ala₂ will be half that of the probe alone because we only detect loss of the probe. The decrease shown in Fig. 2B was in fact slightly more than expected, suggesting that the probe hydrolyzed faster than the natural peptide. At lower concentrations of substrate where \( k \) is constant and \( v \) is a function of substrate concentration, we saw little difference between the competitive and non-competitive rates, as expected for equivalent substrates.

For use of the probes in sediments, we also needed to determine the adsorption behavior of LYA-derivatives since loss of substrate added to sediment can be from both hydrolysis and adsorption. The presence of the LYA tag on the fluorescent probe dramatically decreased adsorption of LYA-alanine (\( K_{ads} \approx 0 \)) compared to alanine (\( K_{ads} = 46 \)). We initially thought that hydrophobicity of the naphthalic moiety would greatly enhance \( K_{ads} \) values of LYA-derivatives, but apparently the sulfonate groups increase water solubility and thus decrease the hydrophobic effect of the naphthalic group. Although we would have preferred that adsorption of the probes be identical to that of natural compounds, solubility of the smaller peptide probes assures that only hydrolysis in pore water is being measured with little interference from adsorptive effects. For longer peptide probes (> 4 amino acids), adsorption must be considered, as for the natural compounds.

Another potential bias in using LYA-peptides to measure hydrolysis rates occurs because few free amine groups are present. LYA attaches to the free N on the terminal amino acid, so that any free N in the LYA-derivative is due to basic amino acids that are part of the peptide chain. Lysis of N-terminal peptides is well known and is the major extracellular or ectoenzymatic proteolysis pathway studied in the marine environment (Chrost, 1991). This is largely because the commonly-used MUF and MCA substrates are cleaved either by aminopeptidases (N-terminal exopeptidases) or by non-specific endopeptidases (that attack interior peptide linkages). However, LYA-derivatives have free C-terminal amino acids and are thus subject to attack by either carboxypeptidases (C-terminal exopeptidases) or endopeptidases. The fact that Leu-MCA was hydrolyzed at rates similar to LYA-dipeptides (Table 3) suggests either that true hydrolysis rates are approximately twice those measured (when C-and N-terminal hydrolyses are summed), or that most hydrolytic activity is due to endopeptidases, and the rates measured by either technique approximate the true rate. The finding that hydrolysis of larger peptides is not sequential, but also not random as discussed later (Figs. 4 and 5), suggests that endopeptidases with a proclivity for specific interior linkages are important. Further studies are required in marine systems to determine the relative importance of exo- vs. endopeptidases.

LYA-derivatives might also not be perfect analogs if the bulky LYA tag sterically hinders enzymatic attack. Peptide hydrolysis rates in seawater decreased in the order \( \text{LYA-ala}_4 > \text{Leu-MCA} > \text{LYA-ala-leu} > \text{LYA-ala}_2 \) (Table 3). Although dipeptides are often more stable than larger peptides (Greenstein and Winitz, 1961), slower hydrolysis of LYA dipeptides might also be expected if hydrolysis of the analogs were sterically hindered. Steric hindrance is difficult to evaluate. The LYA tag does not appear to hinder hydrolysis of peptide bonds as a function of distance from the tag end. The hydrolysis rate constant of each bond in LYA-ala₄ was calculated using the reaction equations in Table 5 for the experiment shown in Fig. 4. Fitting data from the time course experiment with a numerical model (Euler approximation) allowed us to estimate rate constants for the cleavage of each bond in the molecule individually.
Consider a LYA-peptide,

\[ \text{LYA} \rightarrow a_1 \rightarrow a_2 \rightarrow a_3 \rightarrow \cdots \rightarrow a_n \]

where \( k_i \) is the rate constant of the \( i \)th bond (numbered from 1 to \( n-1 \)) of a peptide with \( j \) amino acids (aa) produced after hydrolysis of the original substrate that contained \( n \) amino acids.

The following are the reaction equations for the hydrolysis of LYA-ala:

\[
\begin{align*}
\frac{dC_4}{dt} &= -(k_1^4 + k_2^4 + k_3^4)C_4 \\
\frac{dC_3}{dt} &= -(k_1^3 + k_2^3)C_3 + k_1^3 C_4 \\
\frac{dC_2}{dt} &= k_1^3 C_1 + k_2^2 C_3 - k_2^2 C_2 \\
\frac{dC_1}{dt} &= k_1^2 C_2 + k_2^1 C_3 + k_1^1 C_4
\end{align*}
\]

where \( C_j \) is the concentration of peptide containing \( j \) amino acids. Numerical solution of these equations allows the calculation of \( k_i \).

For the random case, i.e. \( k_i = k \), the equations are:

\[
\begin{align*}
\frac{dC_4}{dt} &= -3kC_4 \\
\frac{dC_3}{dt} &= k(C_4 - 2C_3) \\
\frac{dC_2}{dt} &= k(C_1 + C_3 - C_2) \\
\frac{dC_1}{dt} &= k(C_4 + C_3 + C_2)
\end{align*}
\]

According to the terminology in Table 5, \( k_4^4 = < 0.0001 \text{ h}^{-1} \), \( k_2^4 = 0.22 \text{ h}^{-1} \), and \( k_3^4 = 0.07 \text{ h}^{-1} \), indicating that the second peptide bond is preferentially cleaved. If the presence of the LYA tag were hindering hydrolysis and no other factors were involved, then we might expect \( k_1^4 \) to be higher than \( k_2^4 \). A similar pattern of hydrolysis of LYA-ala_4 and production of LYA-ala_3, LYA-ala_2 and LYA-ala was found in sediments (Fig. 5).

Comparison of rate constants of hydrolysis of LYA-leu-leu, LYA-ala-leu, LYA-leu-ala and LYA-ala-ala in seawater also suggests that steric hindrance from the LYA-tag is not the major influence on peptide bond hydrolysis. The replacement of alanine by leucine (branched amino acid) in the C-terminus position significantly increased dipeptide hydrolysis rate. In spite of leucine being a bulkier molecule, LYA-ala-leu hydrolyzed faster than LYA-ala-ala. LYA-leu-ala cleaves faster than both leu-ala and ala-ala analogs. Such relative rate data suggest that steric hindrance of the LYA tag is less important than differences in peptide structure in triggering preferential cleavage of some peptides over others. Steric hindrance seems to play a much greater role in acid than in enzymatic hydrolysis (Greenstein and Winitz, 1961; Whitfield, 1963). Enzymatic hydrolysis is far more selective, with non-random hydrolysis occurring at particular residues. Although selectivity of enzymes may not be greatly affected by steric hindrance, it is difficult for us to evaluate effects on rate, since currently we can not directly measure hydrolysis of natural peptides at ambient concentrations in seawater or sediments.

4.2. Hydrolysis of LYA-peptides in seawater

Hydrolysis of both LYA-ala_2 and LYA-ala_4 in seawater appears to follow Michaelis-Menten kinetics. This behavior has also been shown for the fluorogenic peptide-like substrates (Hoppe, 1983; Somville and Billen, 1983). Somville and Billen (1983) and Billen (1991) have explored the kinetics of leucyl β naphthylamide hydrolysis in seawater, finding \( K_s = 100 \mu M \) for that substrate. Values found here for the LYA-derivatives, in experiments run at different times, are in the nM range (Table 4), probably closer to natural concentrations of peptides in seawater (see below). Traditionally, half-saturation constants have been interpreted as measures of the affinity for a substrate of an enzymatic system (e.g., Dixon and Webb, 1964) or microorganism assemblage (e.g., Titman, 1976; Billen, 1991). According to this view, the lower \( K_s \) values we found for LYA-ala_4 compared to LYA-ala_4 would suggest that microbial enzymes have a greater affinity for the dipeptide in seawater.

More recently, Button (1993) defined a new "specific affinity" as \( V_m/K_s \). He pointed out that \( K_s \) is directly dependent on \( V_m \), but is insensitive to changes in concentration of enzymes per unit surface area responsible for transport of nutrients across the cell-membrane of microorganisms. In practice, \( K_s \) depends on the shape of the hyperbolic saturation
curve. At low substrate concentration, specific affinity approximately equals the rate constant \((k)\) and is thus a better measure of the likelihood that microorganisms will hydrolyze the compound. Specific affinity values for LYA-ala\(_4\) and LYA-ala\(_2\) (calculated as \(V_m/K_s\)) are comparable to the average \(k\) measured when \(S < K_s\) (Table 4). These values suggest that microbial enzymes have \(\sim 100\) times greater affinity for the tetrapeptide than for the dipeptide, although those experiments were conducted on different days (Table 4). For comparison, in the experiment of 5/7/95 (Table 3), the rate constant for hydrolysis of LYA-ala\(_4\) was also \(\sim 100\) times higher than for LYA-ala\(_2\).

Since natural peptide concentrations in seawater have not yet been reported, we estimated natural substrate concentrations \([S_n]\) using a substrate-addition technique and a plot of turnover time \((\tau)\) vs. concentration of fluorescent substrate (Wright and Hobbie, 1966). The \(x\)-intercept of such a plot is equal to \(K_s + S_n\), while the \(y\)-intercept is the turnover time of the natural substrate. Using the range for \(K_s\) obtained for the fluorescent probes (Table 4), \(S_n\) can be estimated (Table 4). Thus, if we assume LYA-derivatives are true analogs for peptides, ala\(_2\) and ala\(_4\) concentrations in natural seawater at the time of our experiment are estimated as \(\sim 120-180\) and \(0-16\) \(nM\), respectively (Table 4). These estimates of course are a maximum since they include any substrate which competes with the natural or LYA-peptides. Turnover time can be estimated from rate constants; ala\(_2\) turnover time in seawater was thus 1–2 h and ala\(_4\) would completely disappear within a month.

Estimates of \(S_n\) allow us to extrapolate rates of natural peptide hydrolysis in seawater. Assuming that rate constants \((k)\) for the LYA-derivatives (Table 3) and the natural peptides are similar, and using substrate concentrations that include both natural peptide \((S_n\) Table 4) and added LYA-derivative \((70\ nM\) the rate of hydrolysis of ala\(_2\) was \(\sim 1\ nM\ h^{-1}\) and of ala\(_4\) < \(28\ nM\ h^{-1}\). If input rates of the peptides are not vastly different, the faster hydrolysis of the longer peptide could explain its lower ambient concentration. Since it is likely that larger peptides are the source of smaller peptides, further hydrolysis of the smaller compounds would appear to be a rate-limiting step in overall peptide and protein degradation. These peptide hydrolysis rates are not very different from uptake rates of free amino acids measured in adjacent waters. Uptake rates of leucine ranged from 6.3 to 39 \(nM\ h^{-1}\) (winter and summer) in Long Island coastal waters (Pantoja and Lee, 1994); alanine uptake varied from 6.1 to 16 \(nM\ h^{-1}\) during summer (Fuhrman and Ferguson, 1986). Although Fuhrman (1987) estimated that microbial uptake of free amino acids was closely coupled to their production by copepods, our data suggest that hydrolysis of dissolved peptides may also be an important source of free amino acids.

Supporting evidence for the idea that ectoenzymatic plus extracellular hydrolysis of polymers might be the rate-limiting step for microbial decomposition of organic matter is mixed. It has been shown that simultaneous uptake of amino acids was faster than their production by hydrolysis of short peptides in seawater and sediments (Meyer-Reil, 1991; Luo and Henrichs, 1997b). On the other hand, Arnosti et al. (1994) found faster hydrolysis of polysaccharides than uptake of the resulting oligomers in anoxic slurries. They observed accumulation of low-molecular-weight carbohydrates produced during hydrolysis of larger polysaccharides. Our LYA-ala\(_2\) and LYA-ala\(_4\) experiments suggest that the hydrolysis/uptake ratio might vary considerably with substrate, with longer peptides generally hydrolyzed faster than dipeptides.

4.3. Hydrolysis of LYA-peptides in sediment

The turnover time of LYA-ala\(_4\) in sediment pore water was very short. Subsequent production and loss of LYA-peptides occurred in the order LYA-ala\(_3\), LYA-ala\(_2\) and LYA-ala. Loss of substrate from pore water is due to the combined effects of hydrolysis and adsorption onto mineral particles or cell membranes. Adsorption coefficients increased with length of the peptide (Table 2). This increase was also illustrated by a mass balance of the probes in the dissolved phase (sum of substrate plus products). During the first hour when LYA-ala\(_4\) was still present, the sum of substrate and product in seawater was \(\sim 75\%\) of the originally added substrate, as expected from \(K_{ads}\) values. Adsorption coefficients predict that \(\sim 17\%\) of added LYA-ala\(_4\) and \(2\%\) of LYA-ala\(_2\) would be adsorbed. Later in the incubation, when only LYA-ala\(_2\) and LYA-ala were pre-
adsorption of these compounds onto the solid phase was negligible (Table 2).

The sediment hydrolysis experiments of Luo and Henrichs (1997b) found that shorter peptides hydrolyzed faster than longer ones, i.e., \( \text{ala}_2 > \text{ala}_3 > \text{ala}_6 \). We observed the opposite trend: \( \text{LYA-ala}_4 > \text{LYA-ala}_2 \). The difference in relative rates may be due to the differences in the approaches we used. Luo and Henrichs calculated hydrolysis rate based on production of \(^3\)H-free amino acids; they did not separate individual \(^3\)H-labeled peptides in each experiment. If we consider the tripeptide \( \text{ala}_2 \cdot \text{ala}_2 \cdot \text{ala}_5 \) (\( \ast \) denotes the \(^3\)H-labeled amino acid), its hydrolysis can produce either \( \text{ala}_5 \) and \( \text{ala}_2 \cdot \text{ala}_5 \), or \( \text{ala}_5 \cdot \text{ala}_5 \) and \( \text{ala}_5 \ast \) (or both); but hydrolysis is only measured when \( \text{ala}_5 \ast \) is produced since \( \text{ala}_5 \cdot \text{ala}_5 \) and \( \text{ala}_5 \cdot \text{ala}_5 \ast \) are not separated. Therefore, their hydrolysis rate may be underestimated. As the chain length increases, cleavage of more linkages is potentially overlooked. In contrast, our fluorescent LYA-derivatives measure the disappearance of the peptide substrate as well as the production of any fluorescent peptide or amino acid. Differences between relative order of hydrolysis in the two studies could also be due to our use of sediment slurries compared to intact sediments. Previous work has shown that dilution of sediment with seawater affects biochemical reactions like fermentation of amino acids or sulfate reduction (Burdige, 1989). Intrinsic differences between the environments, such as the occurrence of different microbial populations and hydrolytic enzymes, might also be responsible for discrepancies. In particular, different ratios of C- vs. N-terminus dipeptidases might account for differences found between these environments.

### 4.4. Is hydrolysis of peptides in nature random or preferential?

Since we observed peptide hydrolysis rates in seawater and in sediment that varied with size and structure, we investigated whether bond hydrolysis was at random or specific sites. We compared hydrolysis of LYA-ala\(_4\) in seawater with a model in which all bonds are hydrolyzed at the same rate (Table 5). A numerical solution for \( C_1, C_2, C_3 \) and \( C_4 \) was found using an Euler approximation technique; for the case of random hydrolysis, the boundary conditions were \( C_{4(t)} = 55 \text{ nM} \) at \( t = 0 \) and \( C_4 = 0 \) at \( t = 10 \text{ h} \) (the conditions for the experiment shown in Fig. 4). The model solution can be compared with experimental data from the incubation of LYA-ala\(_4\) in seawater (Fig. 4). In the experiment (Fig. 4A and B) the predominant product is LYA-ala\(_2\) with the transient production and subsequent hydrolysis of LYA-ala\(_3\) between 2 and 8 h, and the slow production of LYA-ala after 40 h. LYA-ala production continues for 800 h. A similar pattern was also observed in the incubation of LYA-ala\(_4\) in sediments over a shorter time period (Fig. 5). The random hydrolysis model predicts the simultaneous production of all three peptide products, with subsequent hydrolysis of each to the stable LYA-ala. Thus, the model production of LYA-ala starts at the beginning of the experiment, not after 40 h as we observed experimentally, and reaches the maximum after 15 h instead of 800 h. This exercise illustrates that differential hydrolysis of the peptide analogs occurs, and that bonds are cleaved selectively rather than randomly.

### 5. Conclusions

A new family of fluorescent derivatives was developed to study peptide diagenesis in the marine environment at low substrate levels. These LYA-peptide derivatives undergo extracellular or ectoenzymatic hydrolysis in seawater and in sediments. The fluorescent probes are biochemically similar to their natural counterparts, as demonstrated by competitive inhibition experiments. Hydrolysis of the probes in sediment is primarily in the pore waters because the solubility of the probes limits their adsorption onto the solid phase.

Hydrolysis rates measured in seawater vary with the peptide substrate but are in the same range as hydrolysis rates measured by other techniques. LYA-tetrapeptide hydrolyzed faster than LYA-di-peptides. The fluorogenic substrate Leu-MCA hydrolyzed at rates similar to those of dipeptide derivatives. Estimates of rates of hydrolysis of natural peptides in seawater indicate that peptide hydrolysis could influence free amino acid availability.
Peptide bonds were not hydrolyzed randomly in seawater. A model for random hydrolysis is not consistent with the pattern of hydrolysis observed experimentally.

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