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The effect of chemical structure on the hydrolysis of tetrapeptides along a river-to-ocean transect: AVFA and SWGA

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ABSTRACT

Peptides and proteins are key compounds involved in carbon and nitrogen cycling in biological systems, but little is known about how chemical structure affects hydrolysis rates of these labile compounds in aquatic environments. To investigate effects of chemical structure, custom designed peptides were incubated in waters collected along a salinity transect from the James River, VA, to the coastal Atlantic Ocean. We synthesized tetrapeptide alanine–valine–phenylalanine–alanine (AVFA), a fragment of the common protein ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and its related tri- and dipeptide subunits. We also synthesized a self-designed tetrapeptide serine–tryptophan–glycine–alanine (SWGA). Along the James River–ocean transect, concentrations of added tetrapeptides generally had a 1–2 d lag time before rapidly decreasing, suggesting that microbes needed time to adapt to the pulse of peptide added, being limited by either specific extracellular enzymes or membrane transporters. The hydrolysis rate of SWGA varied significantly along the transect, whereas that of AVFA did not, showing that microbes in different aquatic environments, or their extracellular enzymes, are selective for the peptides they use. Modification of the C-terminal carboxylic acid of AVFA to the α-carboxyamide inhibited its hydrolysis, particularly in relatively oligotrophic marine waters. This suggests that carboxypeptidases are less effective in hydrolyzing peptide amides even though it is apparent that they readily hydrolyze the corresponding peptide acids. This is likely an important process for preservation of peptides existing as amides. Comparison of hydrolysis rates of positionally rearranged tetrapeptides, AVFA, AAVF and AFVA, showed that the sequence of amino acids in a short peptide has little effect on tetrapeptide lability. Modification of SWGA by galactosylation demonstrated that the glycosylation does not confer resistance to short peptides from microbial degradation. Overall, this study shows how structural modifications can influence hydrolysis of short peptides, thus pointing to the need for further research on controls on peptide hydrolysis in the environment.

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1. Introduction

In many marine environments, dissolved inorganic nitrogen limits primary production and thus becomes depleted, especially in surface waters. As a result, dissolved organic nitrogen (DON) is often the dominant N pool in the surface ocean and an important nutrient source to plankton (Bronk, 2002; Mulholland and Lomas, 2008). About 5–10% of the DON pool can be characterized as dissolved combined amino acids (DCAA), and part of this DCAA exists as peptides and proteins (Bronk, 2002). For example, porins and fragments of known proteins have been found in the water column (Tanoue, 1995; Tanoue et al., 1996; Powell et al., 2005; Saijo and Tanoue, 2005). Even though proteins and peptides are a major component in biomass, they are not abundant nor do they accumulate in the environment, suggesting that they either are rapidly consumed by microbes or undergo other rapid transformations.

Once proteins and peptides enter the dissolved organic matter (DOM) pool, they can be easily hydrolyzed into smaller peptides by extracellular enzymes either attached to bacterial cell surfaces (or periplasm) or found free in the water. If less than 600 Da in size, peptides can be taken up directly by heterotrophic bacteria (Weiss et al., 1991). It has been shown that phytoplankton directly take up organic nitrogen compounds such as small peptides (Mulholland and Lee, 2009). Peptides and proteins may also interact with natural organic matter physically through sorption or encapsulation to limit access of extracellular enzymes, thus preserving the protein molecules (e.g., Nagata and Kirchman, 1996; Knicker and Hatcher, 1997; Hedges et al., 2001; Liu et al., 2008). Proteins also have the potential to undergo chemical modification such as Michael-adduct or Schiff base formation, or glycosylation, which can slow their degradation (Keil and Kirchman,

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1993; Yamada and Tanoue, 2003; Hsu and Hatcher, 2005). For example, Keil and Kirchman (1993) showed that ribulose-1,5-bisphosphate carboxylase (RuBisCO) turned over about 100 times more slowly after it was glycosylated. Glycosylated proteins were found to be prevalent in the ocean (Yamada and Tanoue, 2003). Glycosylated proteins are expected to be hydrolyzed eventually into small peptides, some of which may be glycosylated. It is unknown how recalcitrant these glycosylated peptides are compared to the unglycosylated ones. A peptide's terminus (whether acid or amide) may also affect its stability. The C-terminus of many mammalian and insect peptide hormones exists as C-terminal amides like gonadotropin-releasing hormone, oxytocin and vasopressin (Lemke and Williams, 2007). However, the C-terminus of many other peptides is an acid. One important question not previously addressed is how the form of the C-terminus affects hydrolysis of peptides.

Earlier studies estimating rates of peptide hydrolysis mainly depended on fluorogenic substrates such as leucine methyl coumarinylamide (Leu-MCA) and leucyl-β-naphthylamide, which release fluorescent molecules after the internal amide bonds are cleaved (e.g., Hoppe, 1983b; Somville and Billen, 1983; Obayashi and Suzuki, 2005). Another approach is to use ^{14}C labeled protein and follow the appearance of low-molecular-weight hydrolysis products and release of $^{14}CO_2$ from bacterial respiration (Hollibaugh and Azam, 1983). Pantoja et al. (1997) developed an alternative method employing a peptide with an attached fluorophore, Lucifer Yellow Anhydride (LYA), on its N-terminus, allowing the measurement of both the parent compound and the formation of hydrolysis products. Pantoja and Lee (1999) further showed that peptides containing more than two amino acids tend to have much higher hydrolysis rates than dipeptides or Leu-MCA, and they suggested that 1) dipeptidases are not common in nature, 2) other peptidases have low affinity for dipeptides, or 3) the fluorescent tag affects the lability of the peptide bond due to steric hindrance. Steric hindrance by the fluorescent tag is likely. For example, in soil Stevenson (1994) showed that the closer a peptide is attached to an aromatic ring, the less reactive it is. Apparently the use of fluorescence tags has complicated the issue of peptide hydrolysis rates in the marine environment.

In this study, we evaluated the effect of structural modifications on the hydrolysis of two tetrapeptides, alanine–valine–phenylalanine–alanine (AVFA) and serine–tryptophan–glycine–alanine (SWGA) in seawater, on a transect from an estuarine to oceanic environment. Here hydrolysis of peptide includes both extracellular hydrolysis and uptake by cells followed by intracellular hydrolysis. AVFA is a fragment of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO), a ubiquitous protein involved in plant photosynthesis. SWGA is designed to test the effect of glycosylation on its hydrolysis since a galactose molecule can be conveniently attached to the hydroxyl group on the serine (S); W is used to enhance the UV absorbance of the tetrapeptide, and glycine (G) and alanine (A) are two abundant dissolved amino acids (Aluwihare and Meador, 2008). Both AVFA and SWGA contain aromatic groups that can be directly monitored by UV absorbance, thus avoiding the potential steric effects from fluorescent derivatives of these compounds.

Specifically, we synthesized AVFA and its dipeptide and tripeptide subunits, SWGA, and modified structures including AVFA amide, AAVF, AFVA and galactose-SWGA (Fig. A1), using 9-fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (Chan and White, 2000). We measured hydrolysis rates of 1) AVFA and SWGA along an estuarine salinity gradient from tidal freshwater to marine waters; 2) AVFA acid and AVFA amide to determine effects of the C-terminus form on hydrolysis; 3) SWGA and glycosylated SWGA to determine effects of glycosylation on hydrolysis; 4) rearranged tetrapeptides AVFA, AAVF and AFVA to determine the effect of amino acid sequence on hydrolysis, and 5) AVFA, VFA and FA to determine the hydrolysis rates of tetrapeptide subunits. It should be noted that concentrations of these amended peptides were much higher than that of the natural environment, so hydrolysis rates measured here should be considered as potential rates.

2. Materials and methods

2.1. Preparation of peptide standards

Using Fmoc solid phase synthesis (Chan and White, 2000), peptides were synthesized using an automated solid phase peptide synthesizer (PS3, Protein Technologies, AZ). The Fmoc amino acids and reaction solvents [0.4 M N-methyl morpholine in N, N,-dimethylformamide (DMF) for activation, 20% (v/v) piperidine in DMF for deprotection] were obtained from Protein Technologies, and Wang-alanine resin (for peptide acids) and Rink amide resin (for peptide amides) from Novabiochem. All of the peptide syntheses used 0.1 mmol resin (with active reaction sites) and 0.4 mmol Fmoc amino acids. Briefly, the automated procedure included washing and swelling the resin, deprotecting (to remove the Fmoc from the amino group) and activating the first amino acid, and coupling between the resin and the first amino acid in a reaction vessel. This procedure was repeated for each subsequent amino acid addition.

After synthesis, the resin was removed from the reaction vessel, washed with DMF, ethanol, and methylene chloride, and dried in a dessicator under vacuum. The peptide was cleaved from the resin using a cleavage cocktail in a small vial with stirring at room temperature for 2 h. For AVFA, AVFA amide, AFVA, AAVF, VFA and FA, we used a cocktail of 0.5 mL anisole, 0.5 mL MilliQ water and 9 mL trifluoroacetic acid (TFA); for SWGA and galactose-SWGA the cocktail was 8.15 mL TFA, 0.5 mL thioanisole, 0.25 mL 1,2-ethanedithiol, 0.5 g phenol, 0.1 mL triisopropylsilane, and 0.5 mL MilliQ water, according to the Novabiochem online protocols (www.novabiochem.com). After cleavage, the slurry was filtered through a Buchner funnel with fritted glass to remove the resin. TFA in the filtrate was removed by rotary evaporation at 40 °C. The residue in the flask was dissolved in 0.5 mL acetic acid, 4.5 mL MilliQ water, and 10 mL chloroform, transferred to a separatory funnel, and the aqueous layer with the peptide in acetic acid solution was collected. To purify the peptide, the peptide solution was passed through a preparative C_{18} column (Grace, Apollo 5 μ, 150 mm × 10 mm) in a high performance liquid chromatography (HPLC) system with a photo-diode array (PDA) detector and a fraction collector (Shimadzu Prominence). The mobile phases consisted of HPLC grade water with 0.1% TFA, and HPLC grade acetonitrile (ACN) (Fisher) with 0.1% TFA. The flow rate was 1 mL/min, and ACN was ramped from 0% to 100% in 25 min; the gradient was optimized depending on the peptide and on column conditions. By monitoring wavelengths of 254 nm for AVFA and 273 nm for SWGA, peptide peaks were collected using the fraction collector. An aliquot of the collected peptides in ACN and water was used for mass spectrometric analysis (see below) to confirm the product. For the remaining solution, ACN was removed by rotary evaporation at 40 °C, followed by lyophilization. Yields based on the final products purified by HPLC were 30–90%, with higher yields for AVFA and its subunits, and lower yield for SWGA.

To synthesize galactose-SWGA, we used galactosylated Fmoc serine (G-S003, V-LABS) instead of Fmoc serine, and followed the same procedure as for SWGA synthesis. The galactose-SWGA was further treated with 7 M methanolic ammonium for 7 h on an orbital shaker table at room temperature to remove the *o*-acetyl used to protect the hydroxyl groups on galactose (Vuljanic et al., 1996). After removal of the *o*-acetyl groups, methanolic ammonium was removed by rotary evaporation, and the residue was dissolved in distilled water and purified by HPLC as above.

The structures of the synthesized peptides were evaluated by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) and ^1H Nuclear Magnetic Resonance (NMR) spectroscopy. For the MS analysis, formic acid (1%) was added to the peptide solution (approx. 1:1 v/v ACN:H_2O), which was then continuously infused into the ESI-FTICR-MS (Apollo II Bruker Daltonics 12 Tesla Apex Qe) operated in positive ion mode using a modified procedure described previously (Sleighter et al., 2008). For ^1H NMR analysis, 1.5 mg peptide was dissolved in 500 μL D_2O, and analyzed by a 400 MHz Bruker Avance spectrometer (Bruker Biospin, Inc.) using water

suppression (Bruker PRESAT). The obtained spectra agreed well with those simulated by a ^{1}H NMR predictor (ACD LABS 9.0). Based on the HPLC, MS and NMR spectra, the synthesized peptides were determined to be of high purity (>95%) (Figs. A2–4). However, for AFVA and AAVF, the only difference between these two peptides and AVFA was the order of amino acid vials in the peptide synthesizer, so we assume that AFVA and AAVF are of the same quality as AVFA. The MS analysis cannot differentiate between these three peptides because they have the same molecular weight, but the HPLC chromatograms showed that they have different retention times, indicating their successful synthesis.

2.2. Sampling location and description

Water samples were collected in August, 2008, from the tidal fresh to euryhaline James River (JR), the lower Chesapeake Bay (CB, southeast of the JR mouth), and out into the coastal ocean (Fig. 1). These stations covered diverse aquatic environments that included freshwater, brackish water and salt water, as well as highly eutrophic waters (JR and an algal bloom site in the CB) and relatively oligotrophic waters (outside of the Bay) (Table 1). One-liter samples of surface water (~2 m) were collected from 8 L Niskin bottles mounted on a CTD rosette at every sampling station. The sampling station labels refer to the location and salinity, e.g., JR-5 indicates James River and salinity 5.

In April and December, 2008, water was also collected from the shore near the mouth of the Elizabeth River (ER) close to Old Dominion University, Norfolk, VA (Fig. 1), using an acid-cleaned polyethylene bottle. The pH of the water was 7.8 and salinity 17. Dissolved and particulate organic carbon (DOC and POC) were not measured during sampling, but concentrations of suspended particulate matter and dissolved organic carbon at this site are typically in the range of 10–30 mg L^{-1} and 200–250 μM, respectively (Shafer et al., 2004).

2.3. Incubation experiments and peptide measurement

2.3.1. James River transect experiments

The 1-L sample bottles were shaken thoroughly to homogenize samples before subsamples were taken. Aliquots were placed in 50 mL glass flasks (precombusted at 450 °C), and about 40–70 μL peptide standard (in stock solution) was added so that final concentrations were ~10 μM. The samples were immediately incubated in the dark at ambient temperature (~24 °C). A single flask was incubated at each station (In July, we used duplicate flasks and the variation in measured rates was less than 10%). Each flask was sampled after 0, 12, 24, 36, 48, 54, 60, and 72 h. At each time point, the flask was shaken and 1–2 mL water was removed and filtered through a 0.2 μm polyvinylidene fluoride (PVDF, Fisher) syringe filter. The filtered water samples were refrigerated at 4 °C until further analysis within 1–2 d. AVFA and SWGA were incubated at all stations. AVFA amide, AAVF and AFVA were incubated only at stations JR-5 and CB-30. We also incubated SWGA and galactose-SWGA simultaneously using water from station JR-23. The experimental procedure was the same as above except that the water was refrigerated at 4 °C in the laboratory for two weeks before the experiment.

Control experiments using $HgCl_2$ (180 μM) showed that peptide concentration in preserved samples changed little with time. Incubation of SWGA and AVFA under light and dark conditions resulted in no difference in hydrolysis rate.

Temperature, dissolved oxygen and fluorescence were measured *in situ* during the cruise using CTD sensors. Water samples were filtered through precombusted 0.7 μm GF/F filters for fluorometric chlorophyll a (Chl *a*) analysis (Welschmeyer, 1994) or particulate carbon and nitrogen analysis using an automated CHN analyzer (ANCA) (Filippino et al., 2009). Water samples were also filtered through 0.2 μm Supor filters, and the filtrate was placed in acid-cleaned bottles and stored frozen for total dissolved nitrogen (TDN) and for dissolved inorganic nitrogen (DIN: NO_3^-, NO_2^-, NH_4^+) analyses. TDN, NO_3^-, and NO_2^- concentrations were measured using a nutrient auto-analyzer (Astoria Pacific) according to the manufacturer specifications and standard colorimetric method procedures (Valderrama, 1981; Parsons et al., 1984). NH_4^+ concentrations were analyzed manually via the manual phenol hypochlorite method (Solorzano, 1969). DON concentrations were calculated as the difference between TDN and total DIN. Water samples were also filtered through 0.2-μm syringe filters for dissolved amino acid analysis. Dissolved free amino acids (DFAA) were directly measured using high performance liquid chromatography (HPLC) with fluorescence detection after pre-column *o*-phthaldialdehyde derivatization of the samples (Lee et al., 2000). Total dissolved amino acids (TDAA) were determined after the water was

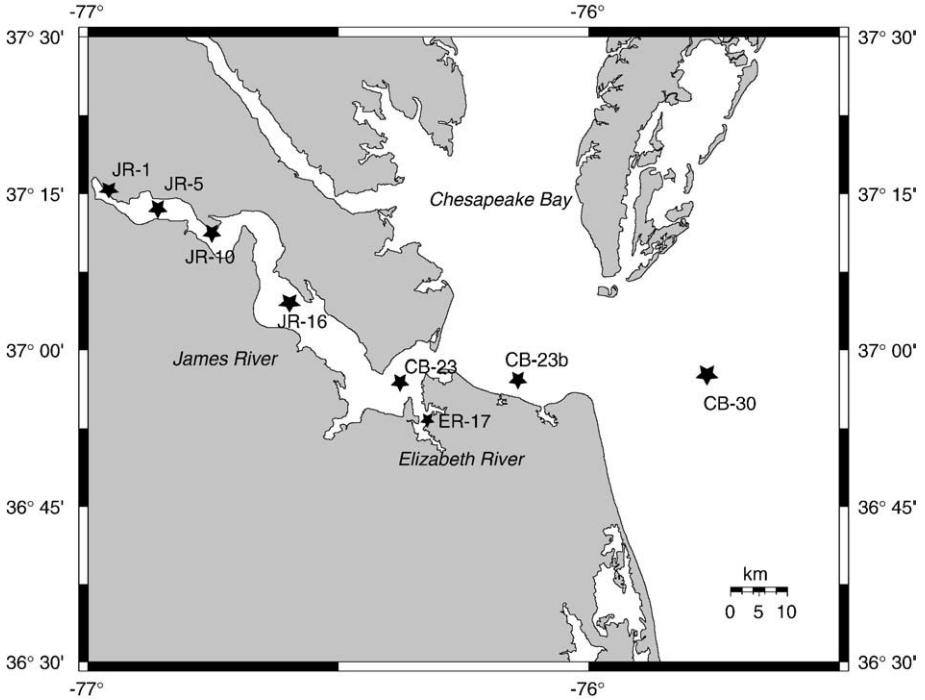

Fig. 1. Sampling stations along a transect from the James River (JR) to the southern Chesapeake Bay (CB). A station near the mouth of the Elizabeth River (ER) close to Old Dominion University, Norfolk, VA, was also sampled. The number after JR, CB, and ER in the station name represents the salinity.

Table 1
Chemical parameters at the sampling stations in the James River (JR) and Chesapeake Bay (CB) in August, 2008.

Station	Salinity	Temp (°C)	Dissolved oxygen (μM)	Chl *a* (μg L^{-1})	DFAA (μM)	DCAA (μM)	PN (μM)	DIN (μM)
JR-1	0.9	27.7	148	17.40 ± 1.13	0.15	0.79	6.31 ± 0.08	2.98 ± 0.76
JR-5	4.6	27.2	127	10.19 ± 0.30	0.16	0.85	18.09 ± 0.67	0.77 ± 0.07
JR-10	9.8	27.5	151	7.91 ± 0.04	0.15	2.19	1.79 ± 0.31	0.74 ± 0.05
JR-16	16.0	26.5	127	2.95 ± 0.40	0.17	2.04	9.69 ± 0.35	8.50 ± 1.02
CB-23	22.6	25.6	164	6.91 ± 0.00	0.60	0.15	14.40 ± 0.97	0.52 ± 0.11
CB-23b[a]	22.9	26.3	198	83.15 ± 0.00	0.32	1.56	28.11 ± 5.96	0.68 ± 0.20
CB-30	29.9	25.4	157	0.26 ± 0.01	0.16	0.80	16.77 ± 0.55	0.16 ± 0.08

[a] At station CB-23b the presence of a phytoplankton bloom was noticed during sampling.

hydrolyzed using a microwave vapor-phase hydrolysis system (Milestone), according to Kuznetsova and Lee (2001) and Kuznetsova et al. (2005). DCAA were determined as the difference between DFAA and TDAA. Amino acid concentrations in replicate samples had relative standard deviations of 10–20%.

2.3.2. Elizabeth River experiment

During April, 2008, water samples from the Elizabeth River were filtered through a 0.2 μm Nylon filter, and 100 mL aliquots were placed in four 125 mL flasks (precombusted at 450 °C). Samples were filtered because of the high load of suspended matter. To inoculate with a natural bacterial assemblage, 1 mL of unfiltered water was added to each flask immediately prior to experiments. AVFA acid and amide standards were added to two of the flasks (final concentrations ~60 μM). Mercuric chloride was added to one AVFA acid and one AVFA amide flask (180 μM) as poisoned controls (Lee et al., 1992; Liu et al., 2006). Samples were incubated for two weeks at room temperature (~24 °C), and 1–2 mL aliquots were taken each day. The sample collection and storage procedures were the same as above for the JR experiments.

Using unfiltered water samples collected in December, 2008, we compared hydrolysis rates of AVFA, VFA and FA in three parallel 30-mL flasks in the dark at room temperature (~24 °C), with final peptide concentrations of 10 μM. A mixture of AVFA, VFA and FA (10 μM of each peptide) was also incubated in a single 30 mL flask to determine the influence of the peptides on each other during hydrolysis. As mentioned above, 1–2 mL water samples were subsampled after 0, 12, 23, 28, 36, 47, 52, 60, 73 and 96 h.

2.4. Peptide quantification

Peptide concentrations in samples from all incubation experiments were quantified using a Shimadzu HPLC system with a C_{18} column (Alltima C_{18} 5 μ, 250 mm × 4.6 mm) and a PDA detector. The mobile phases and gradient program were modified after Pantoja et al. (1997). Briefly, solvent A was 0.05 M NaH_2PO_4 (pH 4.5), and solvent B was methanol, with 1 mL $minute^{-1}$ flow rate. Methanol was ramped from 0 to 80% in 20 min, and then to 100% in 5 min. Quantification was based on absorbance at 210 nm for peptides containing phenylalanine and 220 nm for peptides containing tryptophan. Duplicate sample analyses generally agreed within 5% (relative standard deviation).

3. Results

3.1. Physiochemical properties along the James River transect

The transect samples along the JR to the ocean in August 2008 displayed salinities ranging from 1 to 30 and water temperatures from 25.4 to 27.7 °C, being slightly higher in fresher water (JR-1 to JR-10) than the saltier water (JR-16 to CB-30) (Table 1). All waters were well oxygenated, with concentrations of dissolved oxygen in the range of 127–198 μM. Chl *a* measurements indicated that phytoplankton biomass varied significantly among the stations. Station CB-23b, where a bloom was visible, had the highest Chl *a* concentration (83.2 μg L^{-1}), while station CB-30 had the lowest Chl *a* concentrations (0.3 μg L^{-1}). More moderate Chl *a* concentrations were observed at the other stations (3.0 to 17.4 μg L^{-1}). Similarly, the concentrations of total DIN were much higher at stations JR-1(3.0 μM) and JR-16 (8.5 μM) than at the rest of the stations (all less than 1 μM), with CB-30 having the lowest DIN concentration (0.2 μM). Station CB-23b had the highest particulate nitrogen (PN) concentration, followed by station JR-5 and the two saltier stations CB-30 and CB-23, all above 14 μM; the rest of the stations, JR-1, JR-10 and JR-16, had PN concentrations less than 10 μM. Concentrations of DFAA were relatively constant along the transect, ranging from 0.15 to 0.60 μM, and concentrations of DCAA were in the range of 0.8–2.2 μM. Overall, this JR–ocean transect had physicochemical properties typical of estuarine environments (Bronk, 2002 and references therein), and covered a variety of aquatic environments including fresh, brackish and saline waters, as well as eutrophic and relatively oligotrophic waters.

3.2. Comparison of hydrolysis rates for AVFA and SWGA

In water samples collected along the river–ocean transect, concentrations of added AVFA and SWGA showed a 2-stage hydrolysis pattern (Fig. 2). During stage 1, lasting 1–2 d, peptide concentrations decreased only slightly with time; we call this a lag phase. During stage 2, concentrations of AVFA and SWGA decreased rapidly and generally were undetected after an additional 2 d of incubation. Assuming first-order kinetics, we fit these curves for stage 2 (Tables 2 and 3) (Westrich and Berner, 1984; Pantoja et al., 1997; Kuznetsova and Lee, 2001). The distinction between the two stages is generally apparent, and is defined as the point where the initial concentration decreases by 15%.

For AVFA, the length of stage 1, or the lag time, was 1.0–1.6 d (Table 2). Similarly, lag times of SWGA ranged from 1.0 to 2.2 d (Table 3). Rate constants (k) of AVFA for stage 2 did not vary much among the stations, with the highest at JR-1 (2.0 d^{-1}) and lowest at CB-23 (0.74 d^{-1}) (Fig. 3). In contrast to AVFA, rate constants for SWGA hydrolysis varied significantly among the stations, and were much higher than those of AVFA (Fig. 3). At stations JR-16 and CB-23b (the bloom station), values of k (4.4-5.9 d^{-1}) were 4—5 fold higher than those at other stations (0.6–1.6 d^{-1}).

3.3. Comparison of AVFA acid vs. AVFA amide hydrolysis

Using water from the ER mouth (ER-17) collected in April 2008, concentrations of added AVFA acid and AVFA amide (60 μM) decreased slowly but with no lag time (Table 4 and Fig. 4a). Using single-stage first-order kinetics to fit the hydrolysis curves, the rate constant for AVFA acid (0.14 d^{-1}) is twice that for the amide (0.06 d^{-1}). Both acid and amide preserved with $HgCl_2$ changed little with time.

Using water from the JR–ocean transect, we compared hydrolysis over time of added AVFA acid and amide at JR-5 and CB-30 (Table 2 and Fig. 4b and c). The initial peptide concentration in this experiment was 10 μM, much lower than the ER experiment, so the incubation period was much shorter (3 d vs. 2 weeks). In contrast to the ER experiment, an initial lag time was observed. Concentrations of acid and amide changed little during the initial 24 h. After the lag time, however, concentrations

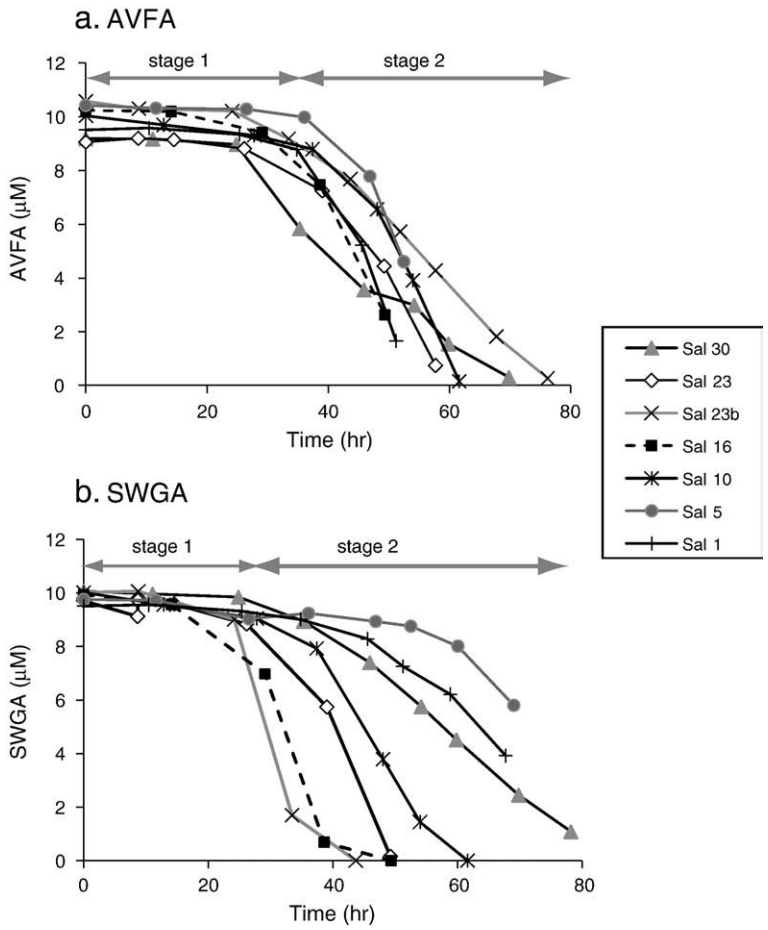

Fig. 2. Concentration changes of the tetrapetides AVFA (a) and SWGA (b) with time in August, 2008. Samples used in the incubation experiments were from the James River transect shown in Fig. 1.

decreased rapidly. At JR-5, AVFA acid decreased slighter faster than AVFA amide, with k values of 1.40 vs.1.04 d^{-1}. At CB-30, however, *k* of the acid (1.14 d^{-1}) was 5 times higher than that of the amide (0.24).

3.4. Comparison of AVFA, AFVA and AAVF hydrolysis

Hydrolysis rates of AVFA, AFVA and AAVF were compared at JR-5 and CB-30 along the JR–ocean transect from August, 2008 (Table 2 and Fig. 5).

Table 2
Rate constants for [a]AVFA and related peptide hydrolysis calculated using first-order kinetics for the period after the lag time.

Station	Initial concentration added (μM)	Lag time (d)	*k* (d^{-1})	R^2
CB-30	9.2	1.03	1.14	0.97
CB-30-amide	9.4	1.03	0.24	0.97
CB-30-AAVF	9.7	1.03	1.00	0.94
CB-30-AFVA	11.1	1.03	0.67	0.97
[b]CB-23b	10.6	1.09	0.95	0.81
CB-23	9.1	1.01	0.74	0.88
JR-16	10.2	1.21	1.54	0.86
JR-10	10.0	1.56	1.43	0.82
JR-5	10.4	1.50	1.40	0.79
JR-5-amide	9.4	1.50	1.04	0.86
JR-5-AAVF	8.3	2.00	1.68	0.86
JR-5-AFVA	10.6	2.00	2.08	0.89
JR-1	9.5	1.45	2.00	0.91

[a] Unless indicated, all compounds including AAVF and AFVA are acid forms. Samples were from the JR and CB in August, 2008.
[b] At station CB-23b the presence of a phytoplankton bloom was noticed during sampling.

As with AVFA at these sites, hydrolysis of AFVA and AAVF with time displayed a 2-stage pattern at both stations. For all three peptides, the lag time at JR-5 was 1.5–2 d, longer than that at CB-30, about 1 d. At JR-5, *k* of AVFA (1.40 d^{-1}) was lower than *k* values of AFVA (2.08 d^{-1}) and AAVF (1.68 d^{-1}). At CB-30, however, *k* of AVFA (1.14 d^{-1}) was slightly higher than *k* values of AFVA (1.00 d^{-1}) and AAVF (0.67 d^{-1}). In a July 2008 experiment, AVFA is also hydrolyzed slightly faster than AFVA at salinity 15 along the same transect (data not shown).

3.5. Comparison of SWGA and galactose-SWGA hydrolysis

Hydrolysis patterns and rates of SWGA and galactose-SWGA were almost identical in waters from station CB-23 (Fig. 6). Both

Table 3
Rate constants for SWGA hydrolysis calculated using first-order kinetics for the period after the lag time. Samples were from the James River (JR) and Chesapeake Bay (CB) in August, 2008.

Station	Initial concentration added (μM)	Lag time (d)	*k* (d^{-1})	R^2
CB-30	10.1	1.03	0.63	0.89
CB-23	9.7	1.09	1.56	0.82
[a]CB-23b	10.0	1.01	4.44	1.00
JR-16	9.9	1.21	5.88	1.00
JR-10	10.0	1.16	1.30	0.86
JR-5	9.8	2.19	0.57	0.92
JR-1	9.5	1.90	0.72	0.95
CB-23-Galactose-SWGA	10.3	0.87	1.30	0.93
CB-23-SWGA	10.5	0.87	1.42	0.94

[a] At station CB-23b the presence of a phytoplankton bloom was noticed during sampling.

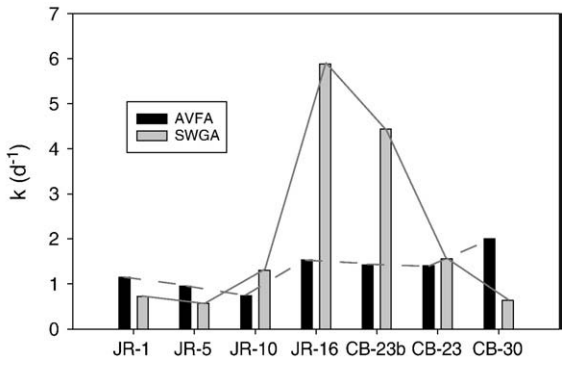

Fig. 3. Rate constants of AVFA and SWGA hydrolysis derived from fits to first-order kinetics.

SWGA and galactose-SWGA had a 0.87 d lag time during stage 1 (Table 3). During stage 2, k was 1.42 d^{-1} for SWGA and 1.30 d^{-1} for galactose-SWGA. We conclude that galactosylation does not significantly affect the hydrolysis of SWGA.

3.6. Comparison of AVFA, VFA and FA hydrolysis

Hydrolysis of AVFA, VFA, and FA, in December, 2008, ER waters (ER-17), were compared in both individual parallel incubations with each peptide added to the sample separately, and in a single incubation with a mixture of these 3 peptides (Fig. 7). Since peptide hydrolysis did not show a clear lag time in these experiments, the curves were fitted with single-stage first-order kinetics (Table 4). In the individual parallel incubations (Fig. 7a), AVFA had a similar rate constant (k) to VFA, 0.48 vs. 0.42 d^{-1}, while the rate constant of FA (0.77 d^{-1}) was nearly twice that of AVFA and VFA. Similarly, when the mixture of these 3 peptides was incubated (Fig. 7b), the rate constant of FA (0.30 d^{-1}) was twice as high as either AVFA (0.18 d^{-1}) or VFA (0.15 d^{-1}). In addition, the rate constants of all 3 peptides were much lower when mixed than in parallel incubations, suggesting that these 3 peptides compete for the same active sites of hydrolytic enzymes.

4. Discussion

4.1. Experimental approach: direct measurement of peptide hydrolysis

The rates of peptide hydrolysis in the marine environment are usually determined using fluorescent amides or peptides labeled with fluorescent tags to increase their detection limits (e.g., Hoppe, 1983b; Somville and Billen, 1983; Pantoja et al., 1997). These techniques rely on release of fluorescent molecules after the amide bonds are hydrolyzed or on hydrolysis of peptides labeled with fluorescent tags, and rate constants determined by these methods include only extracellular enzymatic activity, since the fluorescent molecules or tags cannot be easily taken up by microbes (Pantoja et al., 1997). Peptides tagged with Lucifer yellow anhydride (LYA) further allow one to compare hydrolysis rates of peptides with different numbers of amino acid residues and simultaneously measure hydrolysis products (Pantoja et al., 1997; Pantoja and Lee, 1999). However, peptide hydrolysis rates measured by these techniques may not represent those of natural peptides, which are structurally different from their fluorescent peptide analogs. As suggested by Mulholland et al. (2003), these fluorescent moieties are large chromophores that may hinder enzymatic coupling to the peptides, thus affecting hydrolysis rates, yet this has not been experimentally demonstrated.

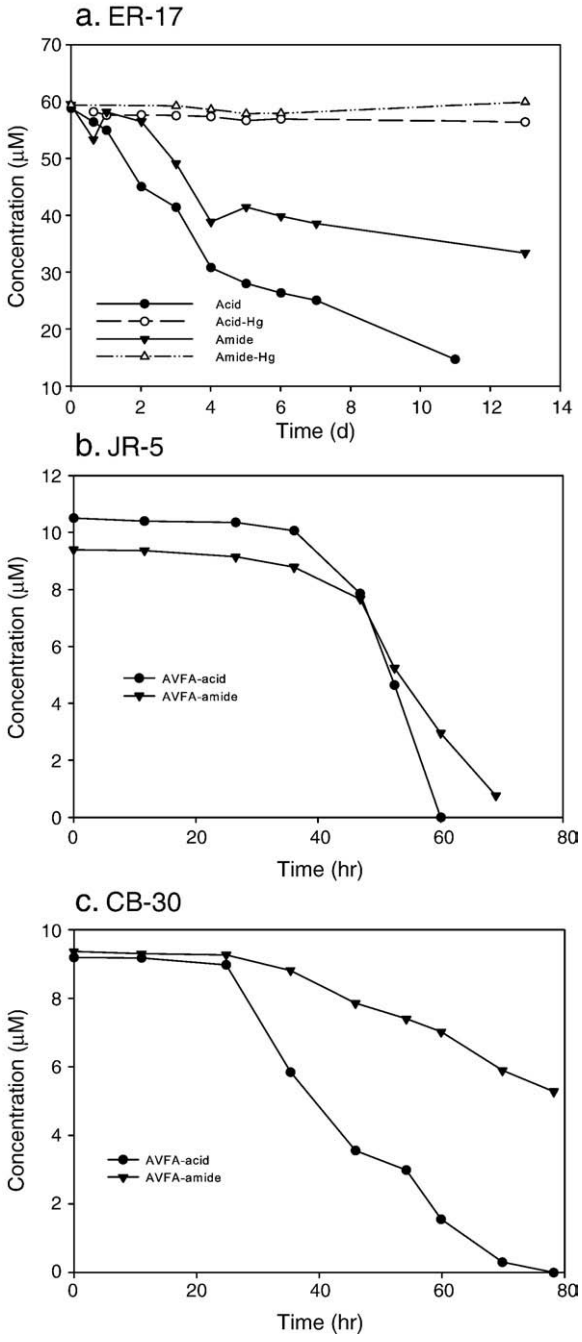

Fig. 4. Concentration profiles of AVFA acid vs. amide as a function of time in waters sampled from a) the mouth of the Elizabeth River, Norfolk, VA. The acid-Hg and amide-Hg are the two control samples poisoned by $HgCl_2$ at a concentration of 180 µM; b) James River at salinity 5; and c) seaward of the Chesapeake Bay at salinity 30 (see Fig. 1 for exact sampling locations).

Table 4
Rate constants for [a]AVFA and related peptide hydrolysis calculated using first-order kinetics. Samples were from the Elizabeth River mouth in April, 2008[1] or December, 2008[2].

Station	Initial concentration added (µM)	Lag time (d)	k (d^{-1})	R^2
[1]ER-17-AVFA	58.8	0.00	0.14	0.98
[1]ER-17-AVFA amide	59.4	0.00	0.06	0.83
[2]ER-17-AVFA	10.0	0.00	0.48	0.92
[2]ER-17-VFA	9.08	0.00	0.42	0.86
[2]ER-17-FA	10.0	0.00	0.77	0.90
[2]ER-17-[b]AVFA	10.0	0.00	0.18	0.95
[2]ER-17-[b]VFA	10.0	0.00	0.15	0.93
[2]ER-17-[b]FA	10.0	0.00	0.30	0.88

[a] Unless indicated, all compounds including AAVF and AFVA are acid forms.
[b] Incubation was conducted with a mixture of AVFA, VFA, and FA at a concentration of 10 µM each.

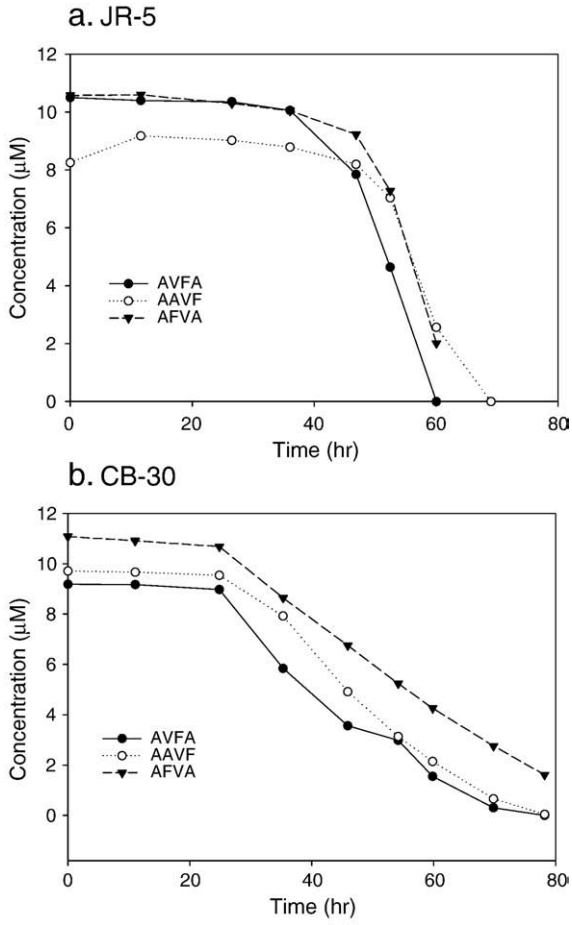

Fig. 5. Concentration profiles for AVFA, AAVF, and AFVA as a function of time, in waters sampled from a) James River at salinity 5; and b) seaward of the Chesapeake Bay at salinity 30 (see Fig. 1 for exact sampling locations).

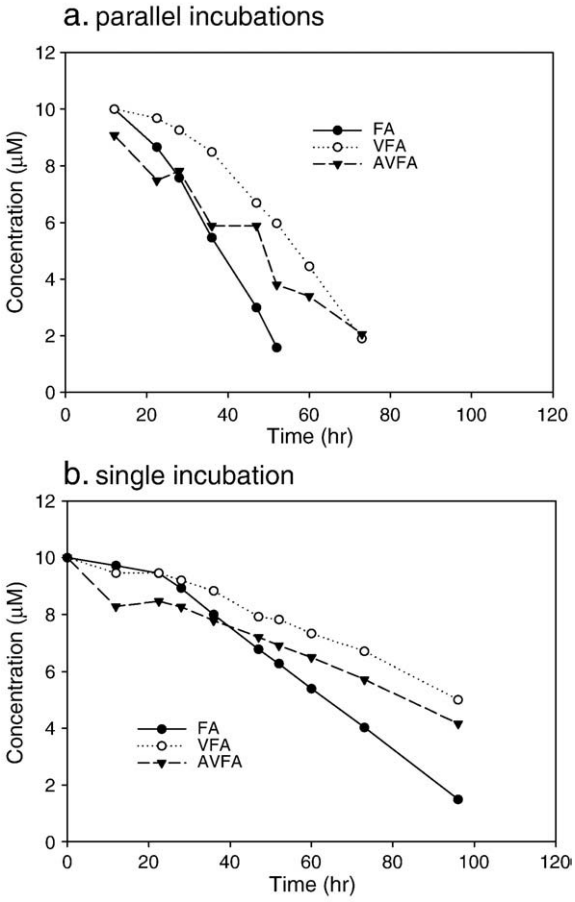

Fig. 7. Concentration profiles of AVFA, VFA, and FA as a function of time using water collected from the mouth of the Elizabeth River, Norfolk, VA. a) Parallel incubations with each peptide and b) a single incubation with a mixture of the three peptides.

The molecular weights of both AVFA (406 Da) and SWGA (420 Da) are less than 600 Da, the size generally thought to limit direct transport across bacterial cell walls (Weiss et al., 1991), so these two peptides are subject to direct uptake in addition to extracellular hydrolysis. Therefore, hydrolysis rates measured here may include both uptake and extracellular enzymatic hydrolysis. The use of non-fluorescent peptides requires addition of higher concentrations (10 μM) than naturally observed (0.2–2.2 μM DCAA), but is necessary for detection purposes. Rather than representing *in situ* hydrolysis rates, our data may reflect the maximum potential hydrolysis rates assuming the hydrolysis of these two peptides follows

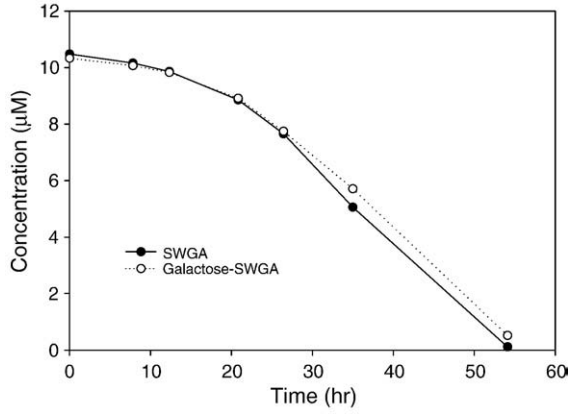

Fig. 6. Concentration profiles for SWGA and galactose-SWGA as a function of time. Water samples were collected from station CB-23 (see Fig. 1).

Michaelis–Menten kinetics (Billen, 1991; Pantoja et al., 1997). Indeed, the concentration used in this study is lower than many previous studies using MCA derivatives, which are often as high as several hundred μM (Hoppe, 1983a; Somville and Billen, 1983; Obayashi and Suzuki, 2005). Kinetics experiments using these fluorogenic substances suggest that hydrolysis rates do not reach a maximum until a substrate level of 100 μM is achieved (Obayashi and Suzuki, 2005). Moreover, the focus of our study is only to evaluate the effect of chemical structure on hydrolysis rates of different peptides, not to measure *in situ* rates, so higher concentrations used in this study should not have a major impact on our results.

In addition to hydrolysis, peptides in our incubation experiments might also be sorbed to particles or colloidal matter, or undergo chemical reaction with DOM (Liu and Lee, 2007; Hsu and Hatcher, 2005). We believe that these processes are not important over the time scale of our experiments. In samples poisoned with $HgCl_2$, concentrations of AVFA and SWGA, changed little during two weeks in ER-sample incubations (Fig. 4a), and the JR-to-ocean transect incubations (data not shown). Sorption of organic molecules to particles generally reaches equilibrium within 2 h (Wang and Lee, 1993; Liu and Lee, 2006) so that a rapid initial decrease in concentration would be expected if sorption were a major pathway for disappearance of the peptides. Chemical reactions between added organic molecules and natural organic matter generally follow second-order kinetics due to the heterogeneity of natural organic matter (Weber et al., 1996), and we observed mainly first-order kinetics. In addition, the fact that these short peptides exist as zwitterions in natural waters may inhibit abiotic reactions of peptide amino groups (Hsu and Hatcher, 2005; Liu and Lee, 2007). Therefore, the 2-stage pattern of disappearance observed in this study suggests that peptide hydrolysis is mainly

through biological pathways including direct uptake and subsequent intracellular hydrolysis, and extracellular hydrolysis by microbes including phytoplankton (Mulholland and Lee, 2009).

4.2. Peptide hydrolysis patterns

Along the JR–ocean transect, hydrolysis of AVFA and SWGA both follow a 2-stage hydrolysis pattern with a clear lag time of 1–2 d regardless of the sampling location. Consistently, numerous studies have shown the existence of a hydrolysis lag time using LYA-tetraalanine (e.g., Pantoja et al., 1997; Pantoja and Lee, 1999; Kuznetsova and Lee, 2001), yet the reason for its occurrence is not well understood. Several factors could affect lag time. It may exist simply because it takes time for certain types of bacteria or extracellular proteolytic enzymes to reach a high enough abundance, or a threshold value, to rapidly hydrolyze the peptides added. Bacterial communities may respond differently to the type of substrate added. For example, Cottrell and Kirchman (2000) showed that in coastal and estuarine environments the *Cytophaga-flavobacter* cluster is mainly responsible for consuming chitin, glucosamine and protein, while α-*Proteobacteria* is more important for amino acids. Thus, the lag time could exist because the extracellular enzymes did not reach the critical concentration to hydrolyze the short peptides.

Another possible explanation for the lag times is that bacteria need time to assemble hydrogel networks to increase the chances of seizing available peptides. The time scale for microorganisms to assemble hydrogels is on the order of tens of hours (Verdugo et al., 2004; Azam and Malfatti, 2007; Ding et al., 2008), consistent with lag times observed in this study. Moreover, if indeed bacteria can take up these short peptides directly, the uptake may be limited by transporters imbedded in bacterial cell membranes (Nikaido and Saier, 1992). Consistently, Arnosti and Repeta (1994) observed that the degradation of galactose–arabinose in anoxic sediments is considerably slower than other disaccharides tested, and they suggested that this disaccharide may not fit the transporters. Alternatively, the initial concentrations of inorganic and organic nitrogen (Table 1) may be high enough to outcompete the "alien" peptides added, which might not begin to be hydrolyzed until the natural substrates are used up. The fact that the lag times vary among the stations suggests that the microbial communities along the transect may be different (Crump et al., 2004; Kirchman et al., 2005; Selje and Simon, 2003; Troussellier et al., 2002).

Interestingly, there is no lag time for the ER samples, even though the sampling locations are only several miles away from the JR transect. Water samples taken from the ER mouth are from shallower depths (intertidal zone, <0.5 m), whereas the transect stations are all from several meters depth. Therefore, sediment might supply different types of hydrolytic enzymes to the water column. Patterns of hydrolysis of polysaccharides are distinctly different between sediment and water, where fundamentally different microbial communities and enzymatic capabilities exist (Arnosti, 2000, 2008).

After the lag times ended, all added peptides disappeared within 2–3 days, with rate constants in the range 1–6 d^{-1}. These rate constants agree well with rates measured using fluorescently-labeled tetrapeptides (Pantoja et al., 1997; Kuznetsova and Lee, 2001; Mulholland et al., 2003), even though the concentrations of tetrapeptides used in those studies are about two orders of magnitude lower than those of the peptides used here. In addition, several studies showed that large protein molecules such as bovine serum albumin and methemoglobin disappeared in 1–3 days (Hollibaugh and Azam, 1983; Billen, 1991; Roth and Harvey, 2006). Keil and Kirchman (1993) observed that hydrolysis rates of RuBisCO along the Delaware estuary averaged 6 ± 3 d^{-1}, in a similar range with those of AVFA and SWGA observed in this study. Overall, AVFA and SWGA are highly labile as expected, and their potential hydrolysis rates in stage 2 fall within the ranges of peptides and proteins used in previous studies.

It should be pointed out that either peptide addition or "bottle effects" could lead to a community structure shift (Cottrell and Kirchman, 2000; Ferguson et al., 1984; Kirchman et al., 2004). Since our incubations lasted about 3 days, the community structure of the bacterial assemblages could shift, which might lead to a two-stage hydrolysis pattern. Unfortunately, the exact mechanism for the lag time remains unclear due to the lack of biological data in this study. These limitations of our experimental design complicate the interpretation of our results for *in situ* processes, but comparisons of the peptide pairs still allow examination of the structural effects on peptide hydrolysis, since incubations were conducted simultaneously under exactly the same conditions.

4.3. Hydrolysis of tetra-, tri- and dipeptides

The small peptides used in this study can be hydrolyzed by extracellular enzymes and/or taken up by microbes directly, as mentioned above. We did not observe the production of hydrolysis products (or indeed any significant additional peaks) in HPLC chromatograms during the incubations of AVFA or SWGA, nor for those of AVF and FA. In AVFA incubations, for example, any hydrolysis product that contains phenylalanine, such as FA, VF, AVF, or VFA, would be observed by the PDA detector due to the UV absorbance of phenylalanine. The fact that we do not observe these intermediate products suggests that both AVFA and SWGA are taken up directly by microbes. Another possibility is that hydrolysis of tetrapeptide and the subsequent uptake of hydrolyzed products by microbes are tightly coupled, i.e., the amino acids, dipeptides, or tripeptides produced from hydrolysis are taken up immediately by microbes without being released into the surrounding waters. There is strong evidence from other studies for such a process, at least under certain conditions such as tight coupling of release and uptake of DFAA in coastal waters (Fuhrman, 1987), or hydrolysis of DCAA and subsequent uptake of DFAA in the sea-surface microlayer (Kuznetsova and Lee, 2002).

The comparison of AVFA, VFA, and FA hydrolysis in December 2008 (ER-17) shows that the dipeptide disappears (either by hydrolysis or direct uptake) much faster than the tri- and tetrapeptide (Fig. 7). Mulholland and Lee (2009) also showed that dipeptides can be taken up as quickly as tetrapeptides. Pantoja and Lee (1999) suggested that dipeptides are extracellularly hydrolyzed more slowly than longer peptides but they could not measure direct uptake since the dipeptide they used was fluorescently tagged (LYA). Thus it is still not clear whether the fluorescent tag inhibits hydrolysis, or whether the tagged dipeptide is simply not taken up directly. If the LYA tag does inhibit hydrolysis, the effect would be greatest for amide bonds closest to the fluorescent moiety, such as LYA-dipeptide or Leu-MCA, whose hydrolysis rates are indeed much lower than those of LYA or MCA tagged with longer peptides (Pantoja and Lee, 1999; Obayashi and Suzuki, 2005).

When incubated individually, the rate constants of AVFA, VFA and FA are about 50% higher than when mixed in a single incubation (Table 4). This indicates that the di-, tri-, and tetrapeptide may compete for the same active sites of either extracellular or cellular transport enzymes present in seawater.

4.4. The effect of chemical structure on hydrolysis of tetrapeptide

Our studies show that the structural form of a peptide can greatly influence its hydrolysis rate. The two tetrapeptides, AVFA and SWGA, had different hydrolysis patterns along the JR–ocean transect: rate constants of SWGA (k: 0.6–5.9 d^{-1}) varied more greatly than those of AVFA (k: 0.7–2 d^{-1}) along the transect, in particular at stations CB-23b and JR-16 (Fig. 3). This difference may be due to the structures of these two tetrapeptides. AVFA contains 4 hydrophobic amino acids, with a hydropathy index of 10.6, calculated by summing the indices of the 4 constituent amino acids (Kyte and Doolittle, 1982). In contrast, SWGA is much more hydrophilic, with an index of only -0.3. This suggests that microorganisms or extracellular enzymes particularly from stations of CB-23b and JR-16 may prefer more polar peptides. Indeed, Roth and Harvey (2006) found that polar amino acids from bovine serum albumin were selectively removed at the initial degradation stage. Further work is needed to test the polarity effect on peptide hydrolysis.

We also saw the effect of structure on hydrolysis rate when comparing peptides with different C-termini. We measured hydrolysis rates of AVFA with both acid and amide termini. The AVFA acid is hydrolyzed consistently faster than the AVFA amide in our incubations using waters of different salinities (Fig. 4), suggesting that peptidases have higher affinity for peptide acid than peptide amide. The difference is especially pronounced at CB-30, where the rate constant of AVFA acid is 5 times higher than that of AVFA amide. This large difference between acid and amide hydrolysis rates is somewhat unexpected since the only difference between them is the C-terminal structure. However, peptidases can be categorized into aminopeptidases, carboxypeptidases, and endopeptidases, and each group has a different specificity. Aminopeptidases cleave peptides at the amino terminus, carboxypeptidases at the carboxyl terminus, and endopeptidases at the internal peptide bonds (Barrett et al., 2004). While all three peptidases can attack the AVFA acid form (with an amino terminus, carboxyl terminus, and internal peptide bonds), only aminopeptidase and endopeptidase can attack the AVFA amide (with only an amino terminus and internal amide bonds). The fact that AVFA acid is hydrolyzed faster than AVFA amide suggests that carboxypeptidase may play a greater role in hydrolyzing peptides in the environment we studied. Billen (1991) suggested that aminopeptidases dominate hydrolysis of natural large proteins in seawater, while Hashimoto et al. (1985) and Obayashi and Suzuki (2005) suggested that endopeptidases and carboxypeptidases are more important. Further work is needed to differentiate the roles of these three types of enzymes in peptide hydrolysis, and to determine whether there is a difference between large proteins and short peptides.

The relative resistance of peptide amide may lead to its preservation in aquatic environments. A fraction of natural protein or peptides exists as peptide amides (Kulathila et al., 1999; Lemke and Williams, 2007), and amide N appears to be the major form of high molecular weight DON in seawater based on ^{15}N solid-state NMR spectra (McCarthy et al., 1996; McCarthy et al., 1997; Aluwihare et al., 2005). It is not clear why amide linkages are so resistant (other than the greater importance of carboxypeptidases mentioned above) and what the exact chemical forms are in DON. Even though peptide amides can eventually be degraded as our incubations showed, their resistance may provide them with more opportunities to escape diagenesis by interacting or reacting with DOM, relative to peptide acids (Hsu and Hatcher, 2005; Yamada and Tanoue, 2003). These results therefore suggest that peptide amides have the potential to accumulate in the DON pool and contribute to the amide linkages found there.

A third test on how structure affects hydrolysis rates was conducted when we rearranged the amino acid sequence of AVFA. Even though AVFA was hydrolyzed slightly faster than its structural isomers AAVF and AFVA (Fig. 5), the differences between rates for these three acids were significant (*t*-test; 95% significance level) only at CB-30, not at JR-5. This suggests that the amino acid sequence in a peptide has little effect on its hydrolysis when the amino acid residues are the same, and that similar enzymes are responsible for degrading these three peptide acids.

Glycosylation is thought to increase the resistance of proteins in DOM to microbial attack (Keil and Kirchman, 1993). Recent studies suggested that a major fraction of proteins in waters is glycosylated (Yamada and Tanoue, 2003; Saijo and Tanoue, 2005). It has been difficult to study the resistance of these glycosylated proteins and peptides and their diagenetic pathways in the marine environment, mainly due to the lack of appropriate standards and analytical techniques that are specific and sensitive to such peptides. Our data show that the hydrolysis rate of synthesized galactose-SWGA is essentially the same as SWGA. Keil and Kirchman (1993), however, observed that the hydrolysis rate of glycosylated RuBisCO was about two orders of magnitude lower than the unglycosylated protein. In a large protein such as RuBisCO, multiple sites, mainly hydroxyl groups from serine and threonine, can be glycosylated with sugar molecules. These multiple adducts cannot only sterically prevent the access of proteases to the protein, but can change the protein conformation and folding (Imperiali and Rickert, 1995; Wu et al., 1999). Both the binding to sugars and the consequential conformational changes are possible inhibitors of protein hydrolysis. For short peptides such as SWGA with only one bound sugar, there is no fixed conformation and the glycosylated compound can rotate freely to allow access by enzymes. This is suggested as a possible explanation for the lack of difference between hydrolysis of the glycosylated and its unglycosylated analog. It is also possible that galactose-SWGA may be directly taken up by microbes, since its molecular weight (582 Da) is just below the threshold size (600 Da) for direct uptake by microbes. Conceptually, large glycosylated peptides are hydrolyzed to mixtures of smaller peptides that are both glycosylated and unglycosylated, and both are likely to be hydrolyzed further at similar rates.

5. Conclusions and implications

In this study, we investigated the effects of peptide structure on peptide hydrolysis. We synthesized two tetrapeptides, AVFA and SWGA, and their tri- and dipeptide subunits, which can be directly measured by UV absorbance due to their natural optical properties. This direct measurement avoids using fluorogenic amides or fluorescently tagged peptides, the conventional way of determining peptide hydrolysis rates. At the same time, the peptides we synthesized are small enough to be taken up directly by cells, so that the potential hydrolysis rates include loss by both extracellular hydrolysis and direct uptake.

By incubating these underivatized peptides in natural waters, we compared the potential hydrolysis rates of several synthesized short peptides in different aquatic environments. All of the peptides incubated along the James River to ocean transect showed a lag time of 1–2 days, suggesting that microbial organisms were not poised to immediately hydrolyze this pulse of labile compounds. Hydrolysis may be limited by specific types of microorganisms, extracellular enzymes, or membrane transporters, and the exact mechanism remains unclear and needs to be further explored. After the lag time ended, however, all of the added peptides were hydrolyzed within 2–3 days, although at different rates; this shows the lability of these compounds. Even though many studies have investigated hydrolysis rates of proteins, and to a lesser extent, peptides, their exact connection to the microbial community is still poorly understood. Questions such as which types of bacteria are responsible for peptide uptake, and how can we differentiate between enzymatic hydrolysis and direct uptake of small peptides less than 600 Da, still remain to be further explored.

Structural modifications to these short peptides affect their hydrolysis rates to different degrees. For example, the acid form of AVFA is hydrolyzed much faster than the amide form, whereas amino acid sequence in the AVFA acid affects hydrolysis rate very little. The resistance of the peptide amide form relative to the acid form may provide clues as to how dissolved proteinaceous matter is preserved. We also found little difference between hydrolysis rates of SWGA and galactose-SWGA. This suggests that the glycosidic bond between serine or threonine and a sugar molecule does not impart resistance to degradation. Once large glycosylated proteins are hydrolyzed into small glycosylated peptides, these peptides can be further hydrolyzed quickly. Our study has just begun to explore the effects of structure on peptide hydrolysis, but has pointed to new approaches that can be used in this area of research.

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Appendix A

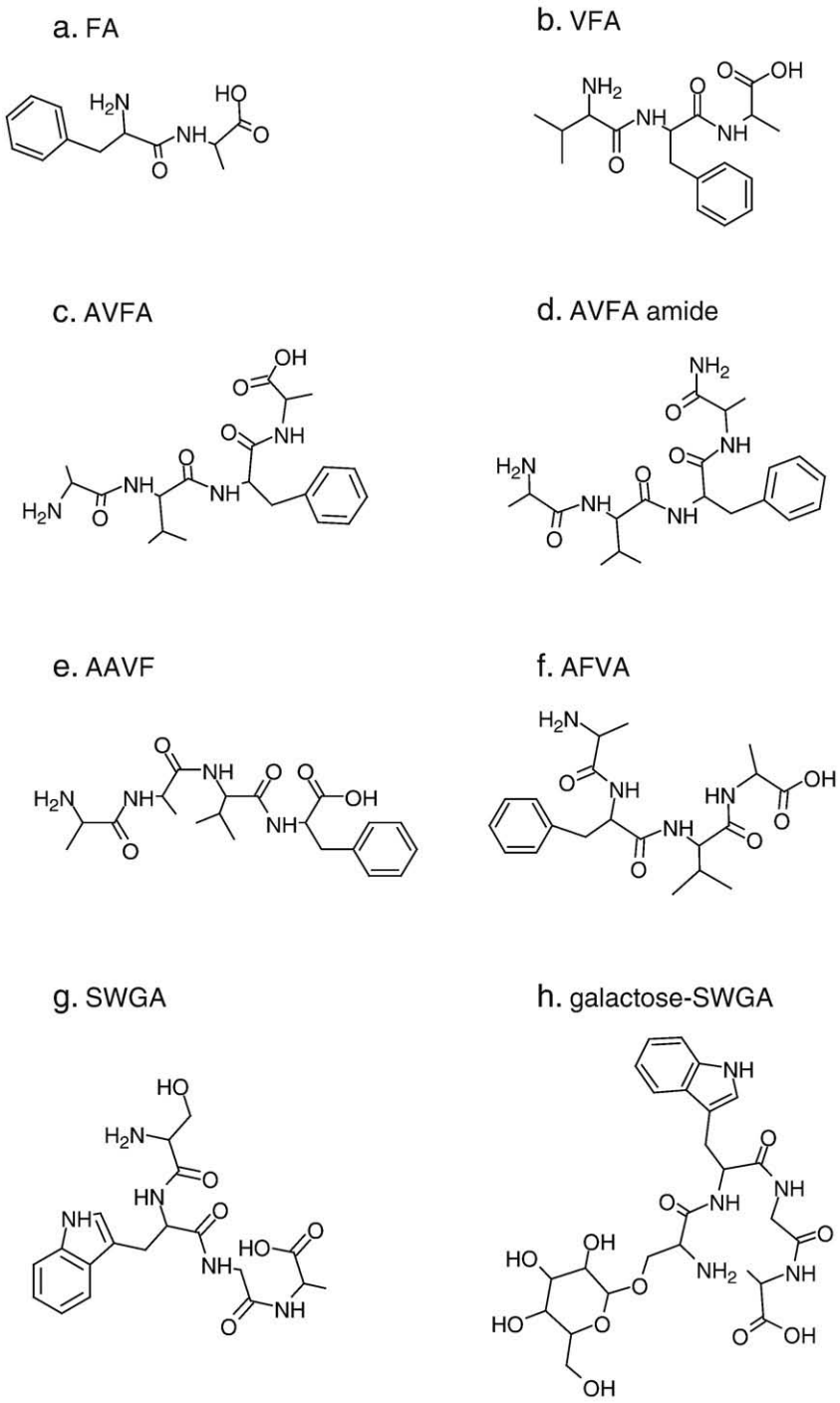

Fig. A1. Chemical structures of synthesized peptides. a. FA ($C_{12}H_{16}N_2O_3$, MW: 236.26758); b. VFA ($C_{17}H_{25}N_3O_4$, MW: 335.39888); c. AVFA ($C_{20}H_{30}N_4O_5$, MW: 406.47694), AVFA amide ($C_{20}H_{31}N_5O_4$, MW: 405.49222), AAVF ($C_{20}H_{30}N_4O_5$, MW: 406.47694), AFVA ($C_{20}H_{30}N_4O_5$, MW: 406.47694), SWGA ($C_{19}H_{25}N_5O_6$, MW: 419.43265) and galactose-SWGA ($C_{25}H_{35}N_5O_{11}$, MW:581.57349). The molecular weights are isotopically averaged neutral masses.

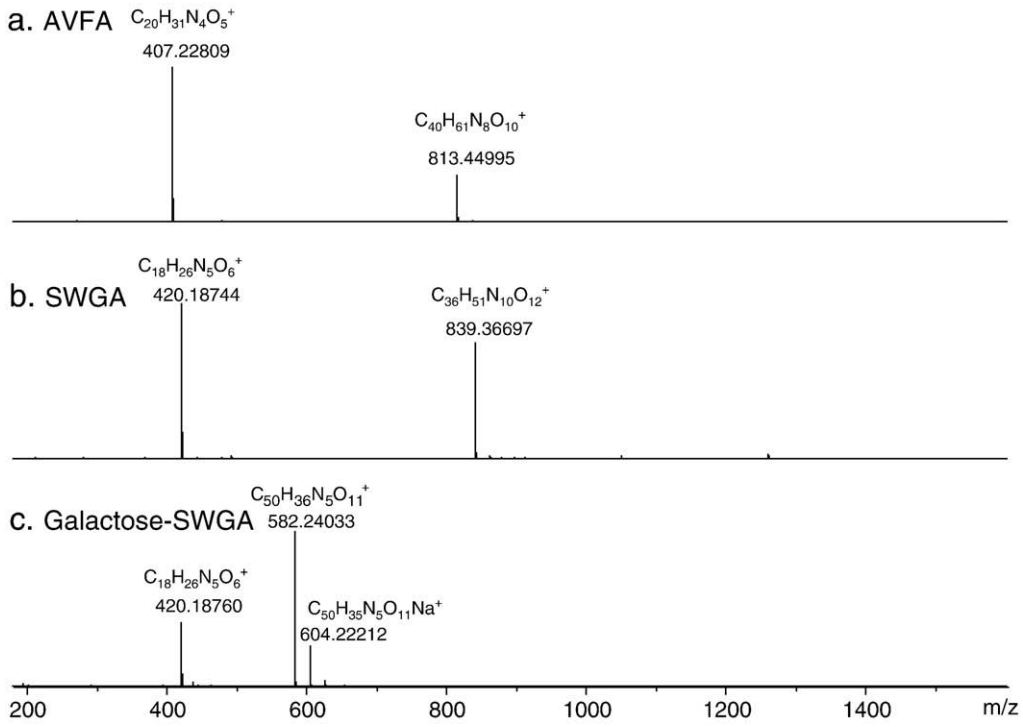

Fig. A2. FTICR mass spectra of synthesized a) AVFA, b) SWGA, and c) galactose-SWGA. The peptides were measured in a positive ion mode (monoisotopic *m*/*z* plus one ^{1}H). The second peaks (*m*/*z* 813.44995 (a), 839.36697 (b)) in the spectra of AVFA and SWGA are their respective dimers. In the galactose-SWGA spectrum, the peak at 420.18760 is the SWGA fragment after electrospray ionization, and the 604.22212 is the sodium adduct of galatose-SWGA. The *m*/*z* of these three peptides measured by FTICR agrees well with the theoretical values, AVFA (error of 2.0 ppm), SWGA (error of 0.8 ppm), and galactose-SWGA (error of 0.4 ppm).

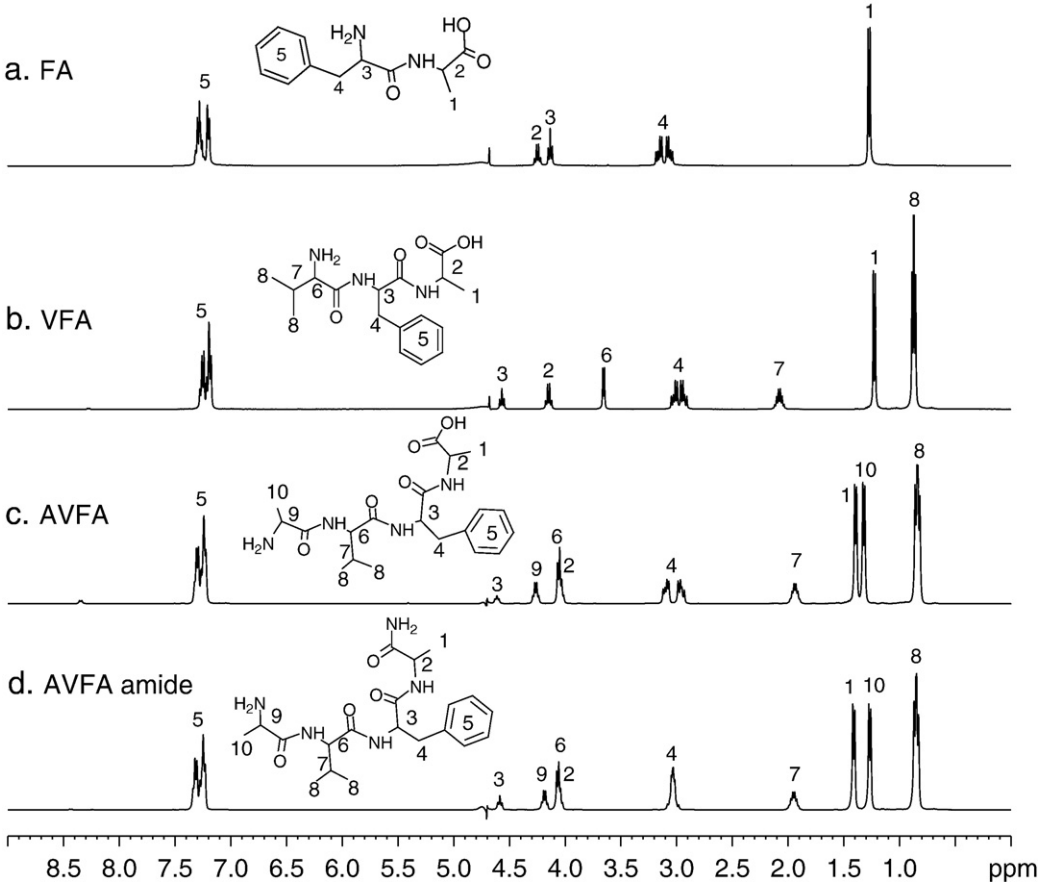

Fig. A3. One-dimensional ^{1}H NMR of a) FA, b) VFA, c) AVFA, and d) AVFA amide. These spectra generally agree well with theoretical simulations (ACD, v. 9.0). The protons in the molecular structures are numbered corresponding with those in the spectrum (for brevity, the Hs on the carbons are not shown in the structures).

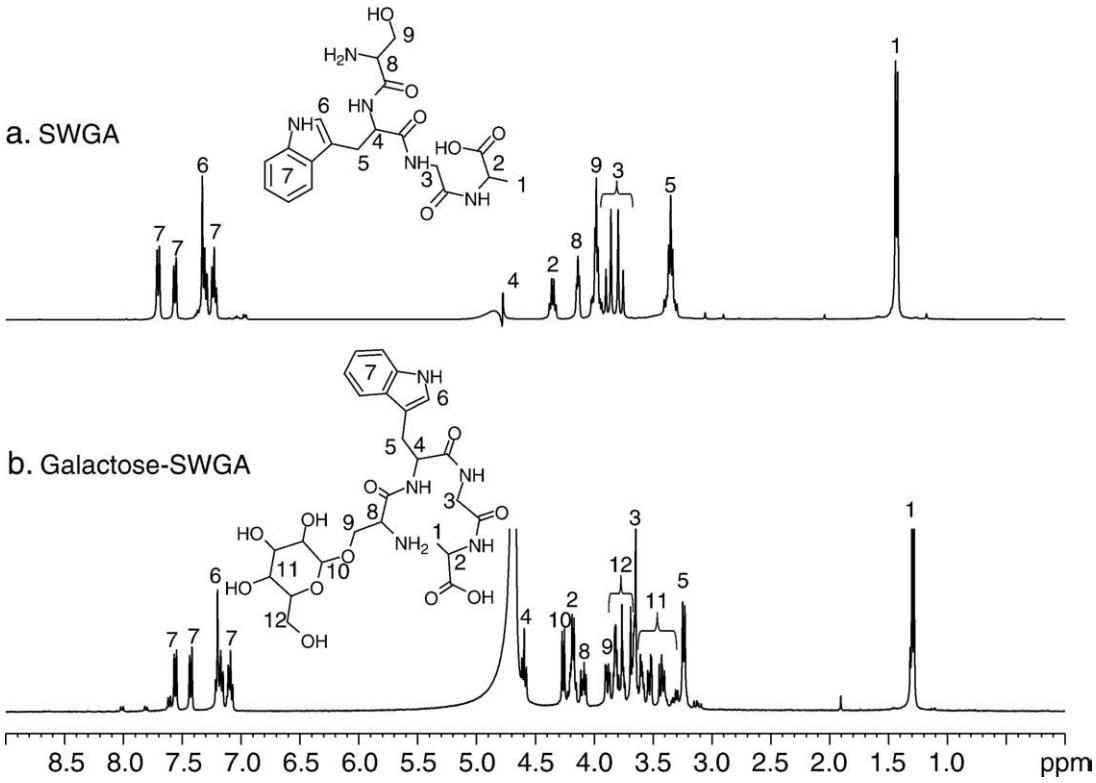

Fig. A4. One-dimensional ^{1}H NMR of a) SWGA, and b) galactose-SWGA. These spectra generally agree well with theoretical simulations (ACD, v. 9.0). The protons in the molecular structures are labeled with numbers, corresponding with those in the spectrum (for brevity, the Hs on the carbons are not shown in the structures).

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