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5 Comparing Extracellular Enzymatic Hydrolysis between Plain Peptides and Their
6 Corresponding Analogs in the Northern Gulf of Mexico Mississippi River Plume

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Abstract

Peptide analogs, such as leucine-methylcoumarinylamide (Leu-MCA) and Lucifer yellow anhydride-tetraalanine (LYA-Ala4), are often used as proxies to quantify extracellular peptidase activities, but how accurately these analogs represent natural peptides remains unclear. Here we compared hydrolysis rates of tetrapeptides alanine-valine-phenylalanine-alanine (AVFA) and tetraalanine (Ala4) with their corresponding LYA analogs along a salinity gradient in the northern Gulf of Mexico Mississippi River plume. Hydrolysis rates were generally similar, but not always, between natural peptides and their analogs in the plume water. For example, hydrolysis rates were similar between LYA-AVFA and AVFA at all stations except one. In contrast, hydrolysis rates of LYA-Ala4 were significantly higher than those of Ala4 at low-salinity (18-19) stations, but significantly lower at one high-salinity (36) station. These results suggest that relative activities of different types of peptidases, measured by the analogs or peptides, depended on biological and chemical properties at each station, such as abundance of certain “opportunistic” bacteria, including *Flavobacterium*, *Ruegeria* and *Roseobacter*. The produced fragments and amino acids from peptide hydrolysis implied further that the hydrolysis pathway depended on specific peptide substrate, and that the LYA tag affected the hydrolysis pathway. Taken together, this study validates the technique of using LYA analogs to study extracellular enzymatic activities, particularly the overall hydrolysis rate, and provides insights into the patterns of extracellular enzymatic activities in estuarine environments.

Key words: plain peptide, peptide analog, hydrolysis, salinity, bacterial community structure, Mississippi River plume, Gulf of Mexico

1. Introduction

As a key step in the cycling of labile organic matter, extracellular enzymatic hydrolysis breaks down proteins or large peptides into small fragments less than approximately 600 Da to allow bacterial uptake in marine environments (Chróst 1991, Weiss et al. 1991). Extracellular hydrolysis involves enzymes that exist either freely dissolved in seawater or as ectoenzymes that are cell-surface bound or in the periplasmic space (Chróst 1990). This process, defined here as peptide hydrolysis, not only regulates the turnover rate of carbon and nutrients (Arnosti 2011), but may also control the types of proteinaceous matter preserved in oceanic dissolved organic matter (Hedges et al. 2000, Aluwihare et al. 2005).

Peptide hydrolysis rates are often estimated using peptide analogs containing fluorogenic tags, such as leucine-methylcoumarinylamide (Leu-MCA) and Lucifer yellow anhydride (LYA)-peptide (Hoppe 1983, Pantoja et al. 1997, Mulholland et al. 2003, Obayashi and Suzuki 2005). Hydrolyzed fluorogenic fragments from the analogs, such as MCA from Leu-MCA, can be determined easily by fluorometry. Targeting certain peptidases, these peptide analogs make it possible to compare peptide hydrolysis rates across different aquatic environments, such as lakes, brackish water, fjords, seawater, marine sediments, and coral reefs (e.g., Hoppe 1983, Mow-
Robinson and Rheinheimer 1985, Hoppe et al. 1988a, Hoppe et al. 1988b, Münster et al. 1989, Rheinheimer et al. 1989). These studies demonstrate the diversity of peptidases and large ranges of enzymatic activities in natural environments.

Despite their extensive application, it remains unclear how representative the peptide analogs are relative to plain peptides without tags, as large fluorogenic tags may affect the enzymatic attack due to the steric effect (Stevenson 1994, Pantoja et al. 1997, Liu et al. 2010). For example, a large tag containing aromatic rings, such as LYA, can increase the rigidity of a

molecule (Wade 1995), thus possibly affecting how the substrate fits into active sites of enzymes. In addition, fluorogenic tags may block either the N or C terminus of peptides from the peptidase attack, while plain peptides can be attacked from either side or the middle (Arnosti 2003). Even though plain peptides are presumably more representative, only a few studies have used plain peptides to study peptide hydrolysis or uptake due to the analytical difficulty in detecting plain peptides in close-to-ambient concentrations (Kirchman and Hodson 1984, Mulholland and Lee 2009, Liu et al. 2010, Liu et al. 2013). Although it has been demonstrated that protein or plain peptide addition can inhibit the hydrolysis of peptide analogs (Payne 1980, Hoppe 1983, Somville and Billen 1983; Pantoja et al., 1997), no studies have directly compared peptide hydrolysis rates between peptide analogs and plain peptides. Our newly-developed liquid chromatography-mass spectrometry (LC-MS) technique makes this comparison possible by measuring peptides in close-to-ambient concentrations (Liu and Liu 2014).

In this study we compare peptide hydrolysis rates between two pairs of plain peptides and their corresponding analogs, Ala4 vs. LYA-Ala4 and AVFA vs. LYA-AVFA, along a salinity gradient in the Mississippi River plume of northern Gulf of Mexico (nGOM). AVFA, a fragment of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and Ala4 are two small peptides with molecular weights of 406 and 302 Da, respectively. The AVFA hydrolysis has been examined in several cases (Liu et al. 2010, Liu et al. 2013, Liu and Liu 2014), but not Ala4. LYA-Ala4 has been applied to measuring hydrolysis rates in marine environments (Pantoja et al. 1997, Pantoja and Lee 1999, Mulholland et al. 2002, Mulholland et al. 2003), but not LYA-AVFA. Owing to the newly developed HPLC-MS technique (Liu and Liu 2014), peptides were amended in close-to-ambient concentrations of total dissolved amino acids (TDAA), which are comparable to those of peptide analogs used previously (Pantoja et al. 1997, Mulholland et al.

2003, Powell et al. 2005, Pantoja et al. 2009). The low concentration of amended peptides may simulate *in-situ* environments better without changing much of ambient organic matter concentrations and the bacterial community structure during short-time incubation (Pantoja and Lee 1999, Kuznetsova and Lee 2002, Liu et al. 2010, McCarren et al. 2010, Liu et al. 2013).

2. Materials and Methods

2.1. Sampling sites

Seawater for peptide incubations was collected from six stations with depths ranging from 17 to 1568 m in the nGOM during a May-2013 cruise on R/V *Pelican*: T1, T2, C6, T3, T6, DWH (Fig. 1 and Table 1). Five stations (T1, T2, C6, T3, T6) were located in the Mississippi River plume along the salinity gradient (Breed et al. 2004), and another station was at the site where Deepwater Horizon (DWH) oil spill occurred in 2010. The oil contamination to the surface water at Sta. DWH was not detectable during the sampling (Liu et al. 2014), so results from this station were likely not affected by the oil spill. Based on the salinity (section 2.2 and Table 1), these six stations were grouped as low-salinity (18-27) stations (Stas. T1, T2, and C6) and high-salinity (35-36) stations (Stas. T3, T6, and DWH).

2.2. Chemical and biological analysis of initial seawater

Surface (2 m) seawater was collected using Niskin bottles mounted on a conductivity-temperature-depth (CTD) rosette (Seabird 911). Simultaneously, *in-situ* temperature, salinity, dissolved oxygen (DO) and chlorophyll *a* were measured by probes in the CTD device (Table 1). Seawater was filtered through 0.2 µm Nylon filters (25 mm dia., Whatman) for analyses of dissolved organic carbon (DOC), total dissolved nitrogen (TDN), TDAA, dissolved free amino acids (DFAA) and nutrients. DOC and TDN were analyzed using a total organic carbon analyzer

(TOC 5000, Shimadzu), and errors between duplicate samples were within 6%. DFAA were analyzed by HPLC (Shimadzu Prominence) with fluorescence detection after pre-column *o*-phthaldialdehyde (OPA) derivatization (Lindroth and Mopper 1979; Lee et al. 2000). TDAA were analyzed in the same way as DFAA but after hydrolysis by 6 N HCl under nitrogen at 110 °C for 20 h (Kuznetsova and Lee 2002). Dissolved combined amino acids (DCAA) were calculated as the difference between TDAA and DFAA. Measurements of replicate samples of DFAA and DCAA had standard deviations of 10-20%. Nitrate and nitrite were measured using the cadmium reduction method, phosphate using ascorbic acid method and ammonium using indophenol method through a UV-Vis spectrophotometer (Evolution 160, Thermo Scientific) (Strickland and Parsons 1968, Jones 1984). DON was calculated after TDN subtracting nitrate, nitrite and ammonium.

For bacterial enumeration, surface water (1 mL) from each station was fixed in 3% (final concentration) formaldehyde and preserved at 4 °C. Bacteria cells were stained with diluted (1:100 v/v) nucleic acid dye SYBR Green II (Molecular probes) and then enumerated under laser excitation at 488 nm with a flow cytometer (BD Accuri C6) following procedures described in Liu et al. (2013). The counting error for duplicate samples was about 12%.

Seawater (0.5-1 L) from each station was filtered through 0.2 µm pore-size Nylon filters for bacterial community structure analysis. The filters were sent to the Research and Testing lab (Lubbock, TX) for pyrosequencing following the procedures described in Liu et al. (2013). The community structure analysis was performed based on bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) method with 16S universal Eubacterial primers Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5'GTNTTACNGCGGCKGCTG for PCR (Dowd et al. 2008, Smith et al. 2010). Tag-encoded FLX amplicon pyrosequencing using a Roche 454

FLX instrument with Titanium reagents in a half plate mode followed the RTL protocols (www.researchandtesting.com). Sequences were classified at the genus level with identity scores between 90% and 95%. Community structures (% genera) of heterotrophic bacteria at all stations were compared by principal component analysis (PCA) using MATLAB® (Xue et al. 2011).

2.3. Peptide incubation

Ala4 and AVFA were custom-synthesized using a solid phase peptide synthesizer by CS Bio. LYA-Ala4 and LYA-AVFA were synthesized through reflux of LYA dipotassium salt (4-amino-3,6-disulfo-1,8-naphthalic anhydride dipotassium salt, Sigma) and Ala4/AVFA in lithium acetate (reagent grade, Sigma), and purified through HPLC according to the protocol of Pantoja et al. (1993). Ala4, AVFA, LYA-Ala4, and LYA-AVFA were incubated on board immediately after surface seawater was collected from two low-salinity stations (T1, T2) and two high-salinity stations (T3, DWH) (Table 1). Each substrate, with a final concentration in the range of 0.23-0.69 μ M that accounted for 4-40% of DCAA (Table 1), was respectively amended in 30 mL amber glass bottles (in duplicate for Sta.DWH or triplicate for Stas. T1, T2 and T3) with 2 mL headspace. The incubation lasted for 24 h in the dark at room temperature (24 °C), which was similar to the *in-situ* temperature (Table 1). At time intervals of ca. 0, 4, 8, 10, 12, 16, 20, and 24 h, duplicate or triplicate aliquots (1.5 mL) were sampled from the incubation bottles, filtered through 0.2 μ m cellulose acetate (CA) syringe filters (13 mm dia., Whatman GD/X) and preserved at -20 °C until analysis. One aliquot (1 mL) from each bottle was fixed with 3% (final concentration) formaldehyde for bacterial abundance analysis.

In addition to the incubations at the 4 stations described above, AVFA, with final concentrations of 0.47-0.50 μ M, was incubated at two other stations, C6 and T6 (Fig. 1). AVFA was incubated in duplicate series of 125 mL amber glass bottles with 25 mL headspace, and

duplicate bottles were sacrificed for sampling at 0, 8, 14, and 24 h, respectively. Incubation waters were amended with 180 μM HgCl_2 to provide killed controls (Lee et al. 1992). Aliquots were taken for peptide analysis at 0, 8, 16, and 24 h following the same sampling protocols.

2.4. Peptide and amino acid analysis

LYA-Ala4 and LYA-AVFA were measured by HPLC with a fluorescence detector (Pantoja et al. 1997). AVFA was measured by HPLC-MS (Liu and Liu 2014). Ala4 was derivatized with *o*-phthaldialdehyde (OPA) and then quantified with HPLC (Pantoja and Lee 1999). Detailed analyses of each peptide and their hydrolyzed peptide fragments or amino acids are described below.

LYA-Ala4 and LYA-AVFA, together with the hydrolysis products including LYA-Ala (or A), LYA-Ala2, LYA-Ala3, LYA-AV and LYA-AVF, were analyzed by HPLC (Shimadzu Prominence) with a fluorescence detector (Pantoja et al. 1997). Mobile phase A (aqueous phase) was 0.05 M sodium phosphate (monobasic anhydrous, ACS grade, VWR) with pH of 4.5, and B (organic phase) was methanol (HPLC grade, Fisher). Samples were eluted through a C_{18} column (Alltima 5 μm , 250 mm x 4.6 mm) maintaining at 40 $^{\circ}\text{C}$ at a flow rate of 1 $\text{mL} \cdot \text{min}^{-1}$. For LYA-Ala4, B was increased from 10% to 30% during the first 12 min, then to 50% at 13 min, and further to 100% at 14 min, and held at 100% for 1 min. For LYA-AVFA, B was increased from 10% to 50% at 25 min, then from 50% to 100% at 26 min, and held at 100% until 32 min. Target peaks of the peptide analogs were detected with a fluorescence detector (Ex: 424 nm; Em: 550 nm), and quantification was based on external standards. Limited by availability, LYA-AV and LYA-AVF were estimated using the response factor (injection amount/peak area) of LYA-AVFA. This assumption is reasonable as the fluorescence was mainly due to the large LYA tag. For example, similar response factors were observed between LYA-AVFA and LYA-A in our

analysis. Authentic standards of LYA-Ala3 and LYA-Ala2 were not synthesized, but LYA-Ala2 and LYA-Ala3 were quantified according to Pantoja and Lee (1999), where similar analytical conditions were used.

Ala4 and its hydrolysis products (Ala3, Ala2 and Ala) were quantified with HPLC after pre-column OPA derivatization (Lindroth and Mopper 1979, Lee et al. 2000). Briefly, the mobile phase A was 0.05 M sodium acetate (HPLC grade, Fisher) with 5% tetrahydrofuran (HPLC grade, Mallinckrodt Baker) and pH 5.7 and B was methanol. For the gradient program, mobile phase B was increased from 20% to 60% during the first 40 min, further to 100% in the next 8 min and then held at 100% for 10 min. Concentrations were quantified by a fluorescence detector (Ex: 330 nm; Em: 418 nm) based on external standards. Since Ala3 overlapped with Ala2 in the HPLC chromatograms, their concentrations were summed using the response factor of Ala3. Note that the summed concentrations might be overestimated because the response factor of Ala3 was higher than that of Ala2, but this problem was not a concern here since their concentrations were close to zero in our samples (see results later).

AVFA and its hydrolyzed fragments were analyzed using HPLC-MS (Shimadzu Prominence) following the protocol of Liu and Liu (2014). In brief, samples were eluted through a C₁₈ column (Alltima 5 μ m, 150 mm x 4.6 mm) with mobile phase A as 10 mM ammonium acetate and B as methanol in a gradient program at a flow rate of 0.3 mL $\cdot$ min⁻¹. A 6-way valve was programmed to direct the sea salt peak to waste before introducing the targeted peaks to the MS detector, which consists of an electrospray ionization (ESI) source and a quadrupole mass analyzer. The MS detector voltage was set at 1.20 kV, interface source temperature at 350 °C and desolvation line (DL) temperature at 250 °C. AVFA and fragments VFA, AVF, VF, FA, AV were analyzed through positive ion mode with selective ion monitoring (SIM) at [M+H]⁺ ion

state of $m/z=407$, 336, 265, 237, and 189, respectively. Amino acids released from AVFA hydrolysis were analyzed by HPLC after pre-column OPA derivatization.

Peptide hydrolysis rates were determined as the slope of the linear regression of hydrolysis curves plotted using concentrations vs. incubation time. Note that the regressions were done only for early time intervals before peptide concentrations became undetectable, typically within 8-24 h. Hydrolysis rates between plain peptides and peptide analogs at each station, or AVFA hydrolysis rates among different stations, were compared through one-way ANOVA ($\alpha=0.05$) and Bonferroni t test ($\alpha=0.05$). Pairwise Spearman rank correlation analysis was applied to the seawater chemical and biological parameters, and AVFA hydrolysis rates were examined at all stations to avoid issues of non-normal data (Kendall, 1990). Significant correlation between environmental parameters and AVFA hydrolysis rates or among environmental parameters was determined using the `rcorr` function in the “Hmisc” package in R.

3. Results

3.1. Chemical and biological properties of the initial seawater

Low-salinity Stas. T1, T2, and C6 were located close to the Mississippi River mouth, whereas high-salinity Stas. T3, T6, and DWH were further offshore (Fig. 1 and Table 1). This salinity gradient corresponds to the unidirectional west/southwest flow of Mississippi River water diluted by oceanic water (Hitchcock et al. 1997, Breed et al. 2004). As expected, chemical and biological properties differed greatly between the low-salinity and high-salinity stations (Table 1). Concentrations of chlorophyll *a* and nitrate in the low salinity stations were more than one order of magnitude higher than in the high-salinity stations. Ammonium, DOC and DON concentrations in the low-salinity stations were about double those in the high-salinity stations except for Sta. DWH. DCAA and DFAA were highest at Sta. T1, and slightly higher at Sta. T2

than at other high-salinity stations except for Sta. DWH. Bacterial abundances in the low-salinity waters were 2-10 times as high as those in the high-salinity waters.

Community structures of heterotrophic bacteria differed greatly between low-salinity and high-salinity waters (Fig. 2 and Table S1). While the low-salinity waters (T1, T2, C6) were enriched in *Flavobacterium* (13-31%), *Ruegeria* (6-10%) and *Roseobacter* (3-11%), the high-salinity waters (T3, T6, DWH) contained mainly *Candidatus Pelagibacteri* (30-42%) and *Sphigobacterium* (9-11%). This difference was examined further in the PCA biplot of major heterotrophic bacterial genus at all stations (Fig. S1). Bacterial communities at the low-salinity sites were clustered clearly as one group and high-salinity ones as the other along PC1, which explained 91% of the data variance.

3.2. Comparison of hydrolysis rates and pathways between plain peptides and LYA analogs

Hydrolysis rates of plain peptides and analogs ranged from near zero in the high-salinity waters to 48 nM·h⁻¹ in the low-salinity waters (Figs. 3, 4, S2, S3 and S4). These rates are consistent with previous incubation studies when similar concentrations of substrates were amended (Pantoja et al. 1997, Pantoja and Lee 1999, Mulholland et al. 2003, Liu and Liu 2014). Hydrolysis rates between plain peptides and the corresponding analogs were similar in 4 out of 8 cases (Fig. 4). LYA-AVFA and AVFA had similar hydrolysis rates at all stations except at Sta. T2. In contrast, hydrolysis rate of LYA-Ala4 was significantly lower than that of Ala4 at Sta. T3 and similar to Sta. DWH, but was higher than those of Ala4 at Stas. T1 and T2. This result was surprising, as we expected that LYA on the N-terminus of Ala4 may block the hydrolysis by aminopeptidases and thus reduce the hydrolysis rate.

LYA-AV and LYA-AVF were the major fragments released during the LYA-AVFA hydrolysis at Stas. T1, T3 and DWH, while LYA-AV was the major hydrolysis products at Sta.

T2 (Fig. 5). LYA-AV accounted for 70%, 53% and 49%, and LYA-AVF accounted for 25%, 42% and 49% of all peptide fragments at the end of LYA-AVFA incubations at Stas. T1, T3, and DWH, respectively. At Sta. T2, concentration LYA-AV was 8 times higher than that of LYA-AVF and 3 times higher than that of LYA-A at the end of incubation. In comparison, dipeptide AV or FA fragments were undetectable during the AVFA hydrolysis at these four stations, and the main hydrolyzed products at all stations were amino acids A, V and F (Fig. 6). A, V and F increased with time during peptide hydrolysis at all stations and then decreased after AVFA was completely hydrolyzed at Stas. T1 and T2. Among the three amino acids, concentrations of F were highest, followed by V and A. Accounting for <10% of hydrolyzed AVFA, VFA and AVF were the only peptide fragments observed (Fig. 6). Produced VFA concentrations were 2-22 times higher than AVF concentrations at Sta. T1, T2 and DWH, while 6 times more AVF than VFA was released at Sta. T3.

Similar to LYA-AVFA, LYA-Ala2 was the dominant fragment throughout the incubation of LYA-Ala4, and accounted for 93-99% of all fragments at the four stations (Fig. S5). During the Ala4 hydrolysis, peptide fragments Ala2 and Ala3 were nearly undetectable, whereas Ala was the only major hydrolysis product (Fig. S6). For AVFA and Ala4, concentrations of total hydrolysis products (use the sum of AVF, VFA and F concentrations for AVFA hydrolysis, and use Ala concentrations for Ala4 hydrolysis) accounted for 52-100% of total hydrolyzed peptides, except <40% for Ala4 at Stas. T1 and T3, and the hydrolysis percentages (concentrations of hydrolysis products divided by initial amended peptide concentrations) were generally higher than the uptake percentages (Figs. S7 and S8).

Concentrations of LYA-Ala4, LYA-AVFA and AVFA in killed controls remained relatively constant during the incubation (Figs. 3, S2, and S3). However, Ala4 concentrations

decreased 26-65%, especially at Sta. T2 (Fig. S4). This decrease was probably due to some free extracellular enzymes that may specifically hydrolyze Ala4, since HgCl₂ does not suppress those extracellular enzymes without sulfhydryl groups (Lee et al. 1992, Liu et al. 2006). This pattern also indicates that the extracellular enzymes that hydrolyzed Ala4 were different than those hydrolyzing other peptides or analogs.

3.3. Changes of bacterial abundances during peptide hydrolysis

At Sta. T1, bacterial abundances during the peptide incubations increased 9-22% from 0 h to 10-12 h, and then increased more rapidly from 12 to 25 h when the peptide hydrolysis was nearly complete (Fig. S9a). The rapid bacterial growth after 10-12 h indicates that bacteria began utilizing the hydrolysis products to build their biomass, and this time frame is consistent with bacterial generation time of 9-12 h (Eilers et al. 2000). At Sta. T2, bacterial abundances in the LYA-Ala4, LYA-AVFA and AVFA incubations increased <11% throughout the incubation, but increased 67% at 20 h in the Ala4 treatment (Fig. S9b). At Sta. T3, bacterial abundance gradually increased by 3-16% from the initial time to the end of incubation (Fig. S9c). Bacterial abundances in the peptide analogs treatments at Sta. DWH increased 18-28% at 24 h, whereas those in the peptide treatments increased 17-25% during 0-8 h and decreased 14-16% afterwards (Fig. S9d).

3.4. Comparison of AVFA hydrolysis along the salinity gradient

In addition to the hydrolysis comparison between plain peptides and LYA analogs at four stations, we further compared AVFA hydrolysis rates at all 6 stations (Fig. S10). AVFA hydrolysis rates differed significantly among all stations except those between Stas. T3 and T6 (Fig. S11, $p < 0.05$). Hydrolysis rates decreased from 13-23 nM·h⁻¹ in the low-salinity waters (T1,

T2, C6) to $1\text{-}5\text{ nM}\cdot\text{h}^{-1}$ in the high-salinity waters (T3, T6, DWH). In contrast, AVFA concentrations remained nearly constant in the killed controls at these 6 stations (Fig. S12), indicating that the decrease of peptide concentrations in the seawater treatments was caused by biological processes instead of physical absorption to glass bottles or particles. Bacterial abundance was highest in the T2 seawater throughout the incubation, followed by T1 and C6 seawater, and then the other high-salinity waters (Fig. S13). Bacterial abundance increased 5-56% during 10-20h in the low-salinity waters, and increased 15-30% during 16-24 h in the high-salinity waters.

AVFA hydrolysis rates along the salinity gradient correlated significantly with salinity (Fig. 7), chlorophyll *a*, DON, nitrate, bacterial abundance and bacterial community structure (use PC1 as the index) in the initial seawater, especially with salinity at a correlation coefficient of -1.00 (Table 2). These environmental factors also co-varied with each other. For instance, significant correlations were found between salinity and chlorophyll *a*, DON, nitrate, bacterial abundance, or bacterial community.

4. Discussion

Concentrations of peptides and peptide analogs generally decreased linearly during short incubation times (Figs. 3, S2, S3 and S4), consistent with previous studies in this region and coastal waters off Port Aransas, TX (Liu et al. 2013, Liu and Liu 2014). This linearity was consistent with a zero-order reaction model, indicating that the hydrolysis was limited by peptidases rather than substrates and that the measured hydrolysis rates approximated enzyme V_{max} according to the Michealis-Mention rate law. If the hydrolysis is limited by substrate, an exponential decrease (first order reaction) would be expected (Pantoja et al. 1997, Stein 2011). This linear pattern also indicates that peptide hydrolysis was not significantly affected by

biological and chemical changes during incubation, such as the changes of bacterial abundance over time (Figs. S9 and S13).

4.1. Comparing hydrolysis rates and pathways between plain peptides and analogs

We expected that plain peptides would be hydrolyzed faster than their corresponding peptide analogs because the tag may block the attack of peptidases due to steric hindrance, which in turn affects how active sites of the enzyme fit into the peptide bonds. For instance, while plain peptides, such as AVFA and Ala4, can be hydrolyzed from both N and C termini by aminopeptidases and carboxypeptidases and from middle by endopeptidases, their LYA analogs are only hydrolyzed by carboxypeptidases and endopeptidases (Stevenson 1994, Pantoja et al. 1997, Liu et al. 2010). However, hydrolysis rates derived from LYA-peptide analogs were generally similar to, or higher than, plain peptide in many cases. For example at Stas. T1 and T2, hydrolysis rates of LYA-Ala4 were higher than Ala4 while LYA-AVFA and AVFA were hydrolyzed at similar rates (Fig. 4).

Plain peptides and their analogs were clearly hydrolyzed through different pathways judged from the hydrolyzed products, assuming that their uptake was minimal during the short incubation. This assumption seems reasonable, as the sum of hydrolysis products typically accounted for ca. 52%-100% of the peptide except for <40% for Ala4 at Stas. T1 and T3, and hydrolysis percentages were generally much higher than uptake percentages (Figs. S7 and S8). The dominance of LYA-AV (Fig. 5), with the simultaneous FA produced during the LYA-AVFA incubation (data not shown), indicates that endopeptidases played a major role in the hydrolysis. Similarly, the major production of LYA-Ala2 suggested that LYA-Ala4 was mainly hydrolyzed by endopeptidases at all the four stations (Fig. S5). Likewise, carboxypeptidases were also important in cleaving LYA-AVFA from C terminus at Stas. T1, T3 and DWH, because

concentrations of LYA-AVF were the second highest among all fragments (Fig. 5). In contrast, AVFA and Ala4 hydrolysis produced mainly free amino acids instead of peptide fragments, suggesting a different hydrolysis pathway from that of peptide analogs (Figs. 6 and S6).

The different hydrolysis pathways between plain peptides and LYA analogs may be caused by the LYA tag. On one hand, the LYA tag may have blocked the attack of aminopeptidases, which could be important for plain peptide as revealed by the high production of VFA fragments during AVFA hydrolysis. On the other hand, the LYA tag may have affected the susceptibility of peptide bonds to peptidases. For example, the dominance of amino acids as hydrolysis products of plain peptides indicated that their sequential hydrolysis was fast and complete, or that all peptide bonds in plain peptides were hydrolyzed by different enzymes in a simultaneous manner. In contrast, the accumulation of LYA-dipeptide products suggests that the peptide bond in LYA-dipeptide was resistant to peptidases, rather than the lack of dipeptidases, as argued by Pantoja and Lee (1999). Otherwise, accumulation of dipeptide would be expected from the hydrolysis of plain peptides. Alternatively, the lack of dipeptide during the hydrolysis may result from direct uptake by bacteria. However, given that the uptake of dipeptides, such as FA and Ala2, is typically slower than that of amino acid A (unpublished preliminary data), the rapid accumulation of amino acids than dipeptides in all incubations indicated that dipeptides were hydrolyzed further into amino acids rather than being taken up directly. Perhaps the steric hindrance of the LYA tag on peptide hydrolysis occurred mostly at the dipeptide bond, because LYA-tetrapeptides were hydrolyzed efficiently to LYA-tripeptides and further to LYA-dipeptides. Therefore, the effect of LYA on hydrolysis may depend on the distance between the LYA and peptide bonds, i.e., steric effects may be higher for closer bonds, as exemplified for LYA-dipeptide. This explanation is consistent with the observation that amino acids directly

attached to aromatic rings are less available to microorganisms than those further away along a peptide chain (Stevenson 1994). Overall, these comparative results provide evidence that hydrolysis of dipeptide is hindered by the steric effects of analog tags.

The relative activities of peptidases differed at each station, which was likely related to biological parameters at each station. For example, while endopeptidases hydrolyzed LYA-Ala4 faster than aminopeptidases and/or carboxypeptidases hydrolyzed Ala4 in the T1 and T2 seawater, they were less efficient in the T3 and DWH seawater (Fig. 4). Ectoenzymes often account for a major fraction of hydrolytic activities in marine environments (Rego et al. 1985, Martinez and Azam 1993, Davey et al. 2001), and these enzymes are mainly synthesized by bacteria, so variations in enzymatic activities among these stations may be caused by different bacterial community compositions (Nagata 2008), as the types and activity levels of hydrolytic enzymes differ among bacterial species (Martinez et al. 1996, Arrieta and Herndl 2002, Arnosti et al. 2005). For example, the cell-specific aminopeptidase activities from 44 marine isolates varied across three orders of magnitude (Martinez et al. 1996). Also, changes of aminopeptidase activity were correlated to bacterial community structure in a mesocosm experiment (Murray et al. 2007). The distinct heterotrophic bacterial community structures observed between low-salinity Stas. T1, T2 and high-salinity Stas. T3, DWH may have caused the variation of relative peptidases activities, as observed consistently (Fig. 2). Perhaps the *Flavobacterium*, *Ruegeria* or *Roseobacter* enriched in the low-salinity waters had higher endopeptidase activities than other bacteria, leading to significantly higher hydrolysis rates of LYA-Ala4 than Ala4 at Stas. T1 and T2. In addition to bacteria, extracellular peptidases can be produced by protists (Karner et al. 1994, Mohapatra and Fukami 2004, Salerno and Stoecker 2009, Thao et al. 2014), which may contribute to the variation of peptidase activities among different stations. However, our

preliminary data showed that the role of protists in peptide hydrolysis at Sta. C6 is limited by comparing peptide hydrolysis between different size-fractionated seawater containing protists vs. without protists in a May-2012 cruise (unpublished data). Whether this holds true for other stations in this area needs more investigation.

4.2. Effects of salinity on peptide hydrolysis

Peptide hydrolysis rates vary in different marine environments, and salinity is often a key environmental parameter that can affect hydrolysis (Roth and Harvey 2006, Mulholland et al. 2003). In our study, chemical and biological parameters differed greatly between the low-salinity and high-salinity waters, including concentrations of organic matter and nutrients, phytoplankton and bacterial biomass, and bacterial community structures (Table 1 and Fig. 2). In these different seawater environments, both peptide analogs and plain peptides were hydrolyzed faster in the low-salinity waters than in the high-salinity waters (Figs. 4 and 7), and hydrolysis rates of AVFA showed a high correlation with salinity (Table 2 and Fig. 7). This pattern may be attributed to the different environmental parameters among the sampling sites, as peptide hydrolysis rate was significantly correlated with chlorophyll *a*, DON, nitrate, bacterial abundance and community structure (using the PC1 index). For instance, higher proportions of *Flavobacterium* were found in low-salinity waters than high-salinity waters (Fig. 2), which is consistent with previous studies (Kirchman et al. 2005, Herlemann et al. 2011, Fortunato et al. 2012, Campbell and Kirchman 2013). *Cytophaga-Flavobacteria* are well known for their capability to degrade biopolymers including proteins (Cottrell and Kirchman 2000, Kirchman 2002, Delong et al. 1993). These *Flavobacterium*, some belonging to opportuni-trophs, which are highly motile and can move

towards high-nutrient conditions (Polz et al. 2006, McBride et al. 2009), could have contributed to the observed higher hydrolytic activity in the low-salinity region.

4.3. Effects of amino acid compositions on peptide hydrolysis

The hydrolysis fragments of LYA-Ala4 and LYA-AVFA suggest that carboxypeptidases preferred to hydrolyze LYA-AVFA than LYA-Ala4, as much less LYA-Ala3 was produced than LYA-AVF (Figs. 5 and S5). This preference of carboxypeptidases to LYA-AVFA was perhaps due to stronger van der Waals interactions between carboxypeptidases and the bulkier amino acids F, V than A (Pantoja and Lee 1999). For example, carboxypeptidase A showed a higher degree of stereospecificity toward the penultimate amino acid from the C-terminus, and the interaction between carboxypeptidase and substrate can be extended to five amino acids beyond the C terminus (Schechter 1970). Carboxypeptidase A also displayed a preference towards penultimate amino acids with aromatic side chains (Smith 1948, Christianson and Lipscomb 1989), so F in LYA-AVFA could fit carboxypeptidases better and thus enhance carboxypeptidase activities relative to LYA-Ala4. Besides carboxypeptidases, substrate composition can affect endopeptidase activities (Obayashi and Suzuki 2005), thus different amino acid compositions between LYA-AVFA and LYA-Ala4 may have caused endopeptidase activities to vary. More peptide substrates with different sizes and compositions are needed to further examine the peptidase specificity on substrates.

5. Conclusion

This study provides the first dataset on the comparison of peptide hydrolysis rates between plain peptides and their corresponding analogs at low concentrations (less than micromolar) in seawater. Several conclusions were drawn from the results:

1. The overall similar hydrolysis rates between plain peptides their corresponding LYA-tetrapeptides suggest that LYA-tetrapeptides are good proxies to study peptide hydrolysis rates. However, their hydrolysis pathways differed due to the steric effects of the LYA tag. Thus, caution is needed when extrapolating comparison results between plain peptides and analogs from one environment to another.
2. Different hydrolysis patterns occur between peptide analogs and plain peptides, which may relate to different relative peptidase activities in seawater environments with different bacterial community structures.
3. The hydrolysis pathway of a peptide can be affected by its amino acid composition, as carboxypeptidases preferably hydrolyzed peptides with bulkier amino acids, such as F and V.

Overall, this study offers insights into hydrolysis rates and patterns of extracellular enzymes in estuarine environments using both plain peptides and their analogs. Different biological and chemical factors among waters with different salinities, together with amino acid compositions in peptides, contribute to different hydrolysis patterns and pathways in estuarine environments.

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Table 1. Environmental parameters of initial surface (2 m) seawater at sampling sites, including coordinates, temperature (Temp), dissolved oxygen (DO), chlorophyll *a* (Chl *a*), dissolved organic carbon (DOC), dissolved organic nitrogen (DON), dissolved combined amino acid (DCAA), and dissolved free amino acid (DFAA).

Site	Coordinates	Temp (°C)	Salinity (ppt)	DO (mg·L ⁻¹)	Chl <i>a</i> (µg·L ⁻¹)	DOC (µM)	DON (µM)	DCAA (µM)	DFAA (µM)	NO ₃ ⁻ (µM)	NO ₂ ⁻ (µM)	PO ₄ ³⁻ (µM)	NH ₄ ⁺ (µM)	Bacterial abundance (cells·ml ⁻¹)
T1	28.97°N, 89.47°W	25.2	18	7.4	1.65	216.7	27.9	5.42	0.30	18.43	0.76	0.31	3.65	1.85*10 ⁶
T2	28.85°N, 89.80°W	25.2	19	6.6	0.82	258.3	35.6	2.95	0.28	15.99	ud	0.11	1.97	8.49*10 ⁶
C6	28.87°N, 90.50°W	25.5	27	7.9	1.51	233.3	12.9	1.79	0.18	0.54	ud	0.11	0.85	1.47*10 ⁶
T3	28.58°N, 90.05°W	24.4	36	6.7	0.08	108.3	8.4	2.88	0.11	0.02	ud	0.11	0.90	8.32*10 ⁵
T6	27.72°N, 90.94°W	25.4	36	6.5	0.04	83.3	6.9	2.34	0.07	0.05	ud	0.11	0.12	3.66*10 ⁵
DWH	28.74°N, 88.36°W	25.1	35	6.6	0.10	325.0	14.8	5.08	0.13	0.03	ud	0.14	0.89	9.19*10 ⁵

ud: under detection limit (ca. 0.03 µM)

Table 2. Correlation coefficients among environmental parameters or between environmental parameters and AVFA hydrolysis rates. Significant correlation was asterisk marked (*, $p < 0.05$; **, $p < 0.001$).

	Temp	Salinity	DO	Chl <i>a</i>	DOC	DON	NO ₃ ⁻	NO ₂ ⁻	PO ₄ ³⁻	NH ₄ ⁺	Bacterial abundance	Bacterial community PC1	AVFA hydrolysis rate
Temp	1												
Salinity	0.33	1											
DO	0.37	-0.47	1										
Chl <i>a</i>	-0.12	-0.93*	0.75	1									
DOC	-0.59	-0.46	0.12	0.43	1								
DON	-0.62	-0.87*	0.20	0.71	0.71	1							
NO ₃ ⁻	-0.12	-0.90*	0.29	0.77	0.14	0.66	1						
NO ₂ ⁻	-0.27	-0.66	0.40	0.65	-0.13	0.39	0.65	1					
PO ₄ ³⁻	-0.68	-0.51	0.19	0.51	0.30	0.44	0.34	0.77	1				
NH ₄ ⁺	-0.50	-0.70	0.32	0.60	0.26	0.77	0.49	0.65	0.51	1			
Bacterial abundance	-0.32	-0.93*	0.41	0.83*	0.60	0.94*	0.77	0.39	0.27	0.71	1		
Bacterial community PC1	0.32	0.93*	-0.23	-0.77	-0.54	-0.89*	-0.89*	-0.39	-0.27	-0.54	-0.94*	1	
AVFA hydrolysis rate	-0.33	-1.00**	0.47	0.93*	0.46	0.87*	0.90*	0.66	0.51	0.70	0.93*	-0.93*	1

Figure Captions

Figure 1. Sampling sites in the northern Gulf of Mexico. Colored isobath contours show depths of the water column. Solid dots, low-salinity stations (C6, T1, T2); open dots, high-salinity stations (T3, T6, DWH).

Figure 2. Major heterotrophic bacterial genus composition (normalized as percentage) of initial seawater at each station.

Figure 3. LYA-AVFA hydrolysis curves with killed control curves (concentration vs. incubation time) at Stas. (a) T1, (b) T2, (c) T3, and (d) DWH. Data points were presented as average $\pm$ standard deviation of replicate samples. Note LYA-AVFA killed control was not conducted at Sta. DWH. Part of the Sta. T2 hydrolysis data was published in Liu and Liu (2014).

Figure 4. Comparison of hydrolysis rates between peptide analogs (LYA-Ala4, LYA-AVFA) and plain peptides (Ala4, AVFA) at Stas. (a) T1, (b) T2, (c) T3, and (d) DWH. Hydrolysis rates were obtained from the slope of linear regression on peptide hydrolysis curves. Error bars represent standard errors of the slope from linear regression. Letters above the bars indicate significant differences (ANOVA and bonferroni t test, $p < 0.05$) of the hydrolysis rates.

Figure 5. Produced peptide fragments (LYA-AVF, LYA-AV, and LYA-A) from LYA-AVFA hydrolysis at Stas. (a) T1, (b) T2, (c) T3, and (d) DWH. Concentrations of LYA-AVF and LYA-AV were calculated by assuming a same response factor between LYA-AVFA and LYA-AVF or LYA-AV from the fluorescence detection. Data points are presented as averages $\pm$ standard deviations of replicate samples.

Figure 6. Produced peptide fragments (AVF and VFA) and amino acids (A, V and F) from AVFA hydrolysis at Stas. (a) T1, (b) T2, (c) T3, and (d) DWH. Data points are presented as average $\pm$ standard deviation of replicate samples.

Figure 7. Comparison of AVFA hydrolysis rates plotted against salinity at 6 stations (T1, T2, C6, T3, T6, DWH). Hydrolysis rates were obtained from the slope of linear regression on peptide hydrolysis curves. Error bars represent standard errors of the slope from linear regression.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

(a)

(b)

(c)

(d)

Figure 5.

Figure 6.

Figure 7.