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Amanda Howard
Bowdoin College

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[1] Dickinson, P. S., Wiwatpanit, T., Gabranski, E. R., Ackerman, R. J., Stevens, J. S., Cashman, C. R., . . . Christie, A. E. (2009). Identification of SYWKQCAFNAVSCFamide: a broadly conserved crustacean C-type allatostatin-like peptide with both neuromodulatory and cardioactive properties. *J Exp Biol*, 212, 1140-1152. [2] Stemmler, E. A., Bruns, E. A., Cashman, C. R., Dickinson, P. S., & Christie, A. E. (2010). Molecular and mass spectral identification of the broadly conserved decapod crustacean neuropeptide pQIRYHQCYFNPISCF: The first PISCF-allatostatin (*Manduca sexta*)

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Quantification and Characterization of AST-C Peptides in *Homarus americanus* Using Mass Spectrometry

Amanda Howard, Class of 2015

Neuropeptides are small signaling molecules found throughout the nervous system that are responsible for influencing animal behavior. They consist of short amino acid chains and interact with cell-membrane receptors in order to regulate behavioral responses (Fig. 1a). The American lobster, *Homarus americanus*, has proven to be a strong model organism in which to study such activity due to the simplicity of the system and the wealth of existing knowledge about the animal. One neuropeptide found in *H. americanus* is a C-type allatostatin (AST-C). Allatostatins are a family of neuropeptides originally identified in insects that inhibits juvenile hormone production. The *H. americanus* AST-C has a pyroglutamate blocked N-terminus and an unmodified C-terminus (Fig. 1b). In addition to AST-C, a different, yet structurally similar neuropeptide has been found in *H. americanus*. This peptide has an unmodified N-terminus and an amidated C-terminus (Fig. 1c). Both forms of AST-C (referred to as ASTC-real and ASTC-like) also have a disulfide bond between their two cysteine residues. In the lobster, both peptides influence cardiac muscle contraction patterns and have been found in various tissues throughout the nervous system [1, 2]. In order to establish the purpose of the observed post-translational modifications, this study aims to find whether these peptides exist in other forms in the lobster and to determine their relative and absolute concentrations.

Liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS) are often used in analytical chemistry to characterize complex samples and identify neuropeptides. First, sample components are separated by chromatography based on properties such as size and hydrophobicity. Using mass spectrometry (MS), peptides are protonated (positively charged) and their mass is determined from their measured mass-to-charge ratios. These peptides are lastly fragmented into many ions using MS/MS, which ultimately allows them to be sequenced in order to determine their identity. This summer, standards of the two AST-C peptides have been characterized by LC-MS/MS. The reduced forms of both peptides have been synthesized by chemically reducing the disulfide bond and were also analyzed by MS/MS. As expected, the structural stability provided by the disulfide bond prevented fragmentation during MS/MS analysis; that is, there was evidence of more fragmentation in the reduced forms than in the fully processed forms (Fig. 2). When looking for other forms of ASTC, these findings will facilitate the identification of the reduced forms in crustacean tissue.

To assess the accuracy of the detection method used, detection limits were assessed by analyzing sample matrices augmented with known amounts of peptide standards. The smallest amount of peptide detected from a single injection was 25 fmol ($2.5 \cdot 10^{-14}$ mol) peptide. There appeared to be a strongly linear relationship between the amount of ASTC-real injected and the instrument response (chromatographic peak area) ($R^2=0.996$, $n=6$). However, the relationship between the amount of ASTC-like injected and the instrument response was less linear ($R^2=0.802$, $n=5$), and the calibration slope was more shallow, indicating that this peptide is more difficult to detect. This is possibly because ASTC-real, unlike ASTC-like, contains an arginine (R) and a histidine (H) residue, two basic amino acids susceptible to protonation. Therefore, it seems that ASTC-real is more easily protonated during the ionization process in MS analysis, causing it to be more readily detected.

Lastly, ASTC-real has been identified in the pericardial organ (PO), a tissue responsible for delivering neuropeptides manufactured in the thoracic ganglion to the heart in order to control muscle contraction. ASTC-like is also believed to be present in the PO based on previous work in the Dickinson lab (E. Dickinson, unpublished data), but it is likely that it has not yet been detected in this study due to the detection limitations described above. To address these issues, more tissues will be pooled to increase the amount of peptide in each sample analyzed.

Currently, tissue extraction methods are being optimized to eliminate phospholipid contamination and to maximize detection sensitivity. Specifically, two separate extraction solvents as well as a chloroform delipidation procedure are being tested. Future goals include quantifying peptide levels by adding a known amount of internal standard to the samples and comparing instrument responses for ASTC and for internal standard. Additionally, known amounts of peptide standard will be brought through the extraction process to determine the amount of peptide loss throughout this procedure. During the upcoming academic year, this study will be continued as an Honor's project. Further research in these areas will ultimately help explain how neuropeptides interact to regulate behavior within the lobster and in more complex systems.

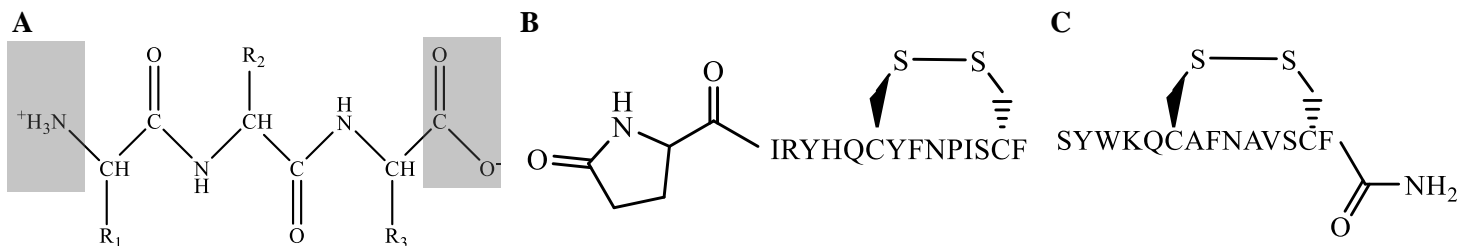


Figure 1. (a) Basic peptide structure. N-terminus on left (shaded) and C-terminus on right (shaded). The identity of each amino acid is determined by its unique R-group (i.e. methyl (CH₃), ethyl (CH₂CH₃), etc.). **(b)** ASTC-real structure. The fully processed form as found in *H. americanus* has a pyroglutamate group at the N-terminus and an unblocked C-terminus. Each letter represents a specific amino acid. **(c)** ASTC-like structure. The fully processed form as found in *H. americanus* has an amine group at the C-terminus and an unblocked N-terminus.

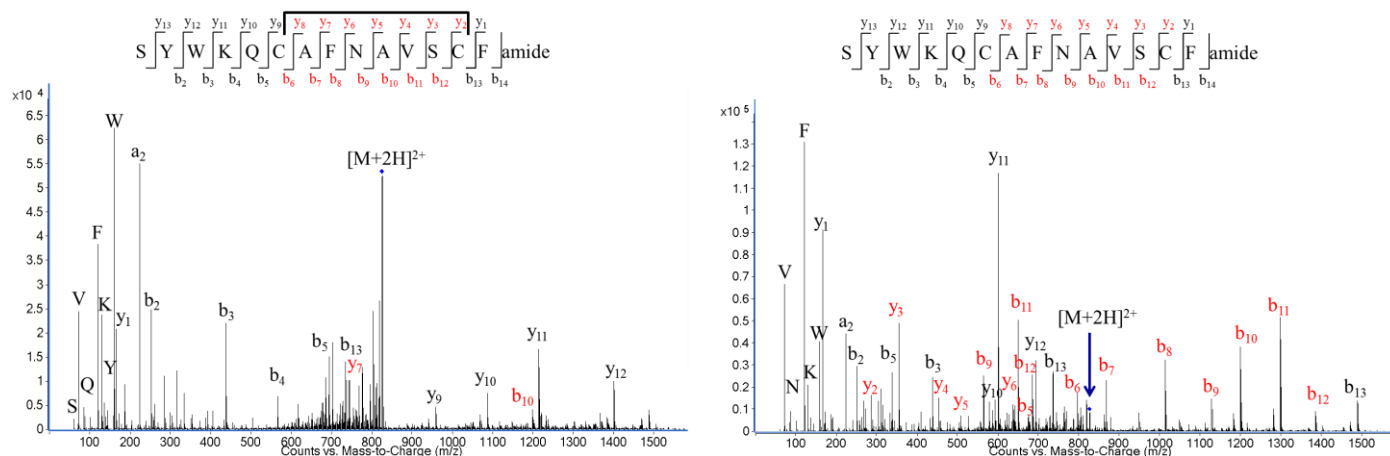


Figure 2. (a) MS/MS spectrum for ASTC-like standard in its fully processed form with a disulfide bridge connecting both cysteine (C) residues, as seen in *H. americanus*. Ions formed by fragmentation at sites between the cysteine residues labeled in red. Only two of these ions were clearly detected. **(b)** MS/MS spectrum for reduced ASTC-like (no disulfide bond). As expected, there was more fragmentation in the reduced form than in the oxidized form. More ions were detected after the disulfide bond reduction, particularly those formed by fragmentation between the two cysteine residues (red).

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References

- [1] Dickinson, P. S., Wiwatpanit, T., Gabranski, E. R., Ackerman, R. J., Stevens, J. S., Cashman, C. R., . . . Christie, A. E. (2009). Identification of SYWKQCAFNAVSCFamide: a broadly conserved crustacean C-type allatostatin-like peptide with both neuromodulatory and cardioactive properties. *J Exp Biol*, 212, 1140-1152.
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