Optimization of Large Dense Core Vesicle Purification for Mass Spectrometric Detection of Neuropeptides

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Objectives

1. Purify large dense core vesicles (LDCVs) from neurons directly:
   1) Optimize continuous sucrose gradients using manual preparation
   2) Evaluate the efficacy of different protease inhibitors
2. Compare sequence-determined properties of neuropeptide (NP) identifications from conventional and LDCV extraction

Introduction

Neuropeptides are synthesized in neurons for long and short range signaling. Mature NPs are stored in LDCVs for controlled release upon stimulus.

- Characterize to understand modulation of life processes.
  - Challenging due to high sequence diversity and low abundance in vivo.
- Extraction for mass spectrometric detection:
  - Popular to extract from entire neuronal ganglia; highly abundant cell debris.
  - We propose an optimized extraction from purified LDCVs.

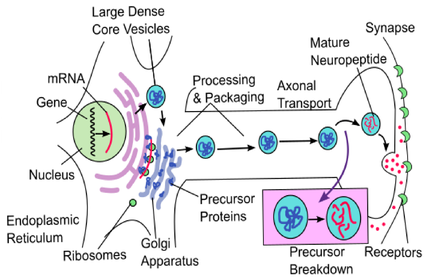


Figure 1. NPs are synthesized in the cell soma and transported through axon in LDCVs for release upon stimulus.

Sucrose Gradient Optimization

Two manual preparation methods:

1. Freeze-Thaw: Layer decreasing sucrose concentrations, freeze to control mixing. Thaw/diffuse for 8 – 24 hours.
2. Horizontal Diffusion: Layer two sucrose concentrations. Tilt horizontally for 1.5 – 4 hours to diffuse.

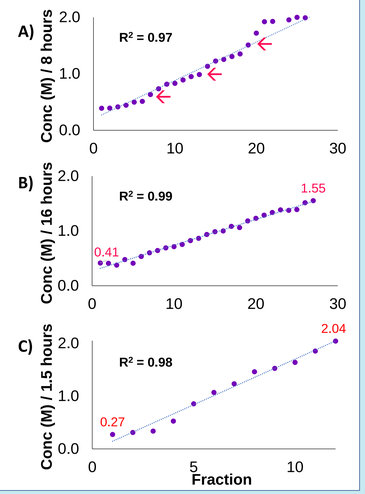


Figure 2. Sucrose concentration linearity of gradients prepared by freeze-thaw layering of **A)** four or **B)** seven solutions and **C)** horizontal diffusion of two solutions were evaluated via UV-Vis spectroscopy. The most optimal gradient produced by each method is shown.

- Freeze-thaw led to discontinuity (←) and over diffusion.
- Horizontal diffusion led to desired continuous gradients in a desired time frame.

Meta-Analysis

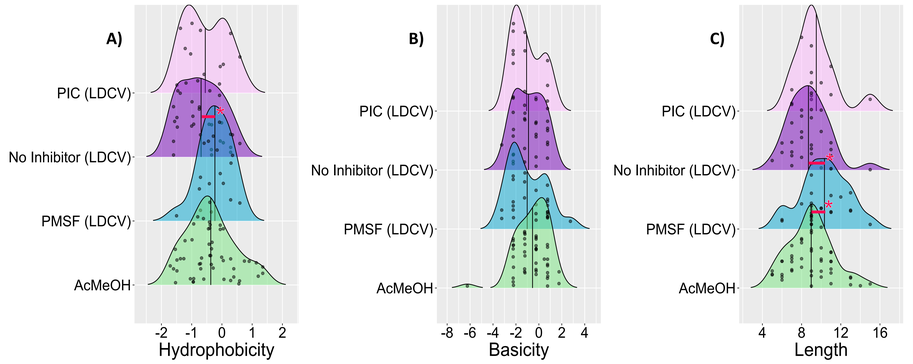
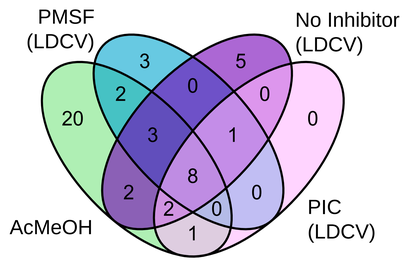


Figure 4. Ridgeline plots of neuropeptides **A)** *KyteDoolittle* hydrophobicity scores, **B)** *EMBOSS* basicity scores, and **C)** sequence lengths for extractions from purified LDCVs (in solutions of PMSF, PIC, or no inhibitor) and conventional extractions using AcMeOH. P-values < 0.05 (*) are statistically significant.

Protease Inhibitor Optimization

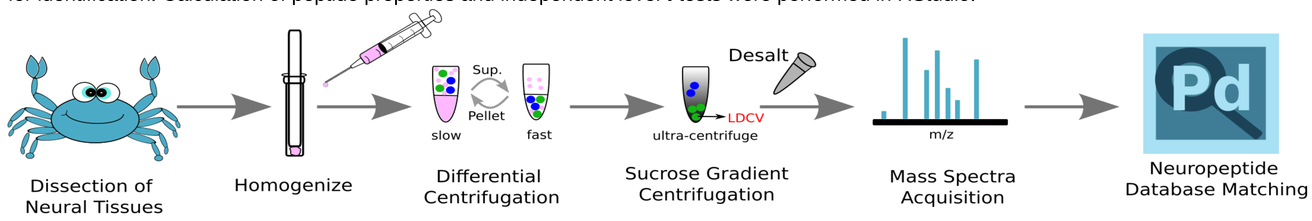


- 12, 17, and 21 NPs were identified from LDCV purifications using PIC, PMSF, and no inhibitor, respectively.
- No unique NPs were observed using PIC.
- 38 NPs were identified from conventional extraction in acMeOH.
- Extraction in acMeOH recovered 20 unique NPs.

Figure 3. NPs identified from each condition.

Methods

Thoracic ganglia were manually homogenized in solutions containing phenylmethylsulfonyl fluoride (PMSF), Pierce Protease and Phosphatase Inhibitor cocktail (PIC), or no inhibitor. LDCVs were purified via differential and sucrose gradient centrifugation[1]. Control samples were homogenized and extracted in acidified methanol (acMeOH). Desalting was performed with a C18 ZipTip. Data was collected using an orbitrap mass spectrometer and analyzed via Proteome Discoverer and an in-lab built crustacean NP database for identification. Calculation of peptide properties and independent level t-tests were performed in RStudio.



1. Birinci, Y., Preobraschenski, J., Ganzella, M., Jahn, R., & Park, Y. (2020, May 5). *Isolation of large dense-core vesicles from bovine adrenal medulla for functional studies*. Nature News. https://www.nature.com/articles/s41598-020-64486-3.

Conclusions

- Horizontal diffusion produced optimal continuous sucrose gradients.
- Most NP identifications discovered using conventional extraction in acMeOH.
  - LDCV purifications only recover unreleased and mature NPs; acMeOH additionally recovers immature and exocytosed NPs.
- NPs extracted from LDCVs in PMSF showed statistically different property trends from other purification techniques:
  - More hydrophobic than the no inhibitor method.
  - Longer than those from no inhibitor and acMeOH methods.
  - Inhibitors prevent degradation of receptor protein that would stop proteolytic processing.

Future Directions:

- LDCV purification could increase confidence that sequences are not degradation products, post-translational processing has only occurred in vesicles, and NPs were synthesized from local tissue (i.e., did not travel to act on foreign receptors).
  - NPs released from degraded vesicles are not recovered using LDCV purification.
- LDCV purification can be used to distinguish local and acting NPs from different ganglia (conventional extraction cannot distinguish between pre- and post-synaptic NPs).
- Additional replicates with larger pools of tissue should be used to detect more NPs and confirm they are bona fide.

Acknowledgements

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