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Characterization of peptides from *Aplysia* using microbore liquid chromatography with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry guided purification

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Abstract

Liquid chromatography (LC) has been used extensively for the separation and isolation of peptides due to its high selectivity and peak capacity. An approach combining microbore LC with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-MS) detection is described to identify peptides in cells and guide the purification of peptides from the marine mollusc *Aplysia californica*. Direct MALDI-MS of neurons and processes provides molecular mass information for unknown peptides with almost no sample preparation, and LC–MALDI-MS allows the isolation and purification of these peptides from pooled samples, thus enabling new putative neuropeptides to be isolated from complex cellular samples. Both direct MALDI-MS and LC–MALDI-MS are compared in terms of detecting peptides from neuronal samples. Using both approaches, two peaks from *Aplysia californica* connectives having molecular masses of 5013 and 5021 have been isolated, partially sequenced and identified as novel collagen-like peptides.

Keywords: Liquid chromatography–mass spectrometry; Matrix-assisted laser desorption/ionization mass spectrometry; *Aplysia californica*; Peptides; Neuropeptides; Collagen

1. Introduction

The ability to relate the activity and plasticity of neurons to behavior is an important goal of neuroscience. Because of the complexity of the mammalian central nervous system (CNS), this is a difficult and challenging task. An advantage of the marine mollusk *Aplysia californica* to such neuroscience research is its much simpler CNS consisting of approximately 10 000 neurons contained in groups called ganglia. Many of the neurons are relatively large and are identifiable based on morphology, location, biochemistry, and even have relatively invariant neuronal connections. This makes many investigations into the biochemistry, molecular biology and behavioral neuroscience tractable in *Aplysia* [1–6].

We are interested in understanding the interplay of neurotransmitters and neuro-modulatory peptides in several identified neurons, as well as in elucidating new neuroactive peptides in *Aplysia*. A number of

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factors make studying peptides difficult; compared to classical neurotransmitters, neuropeptides are present and active at lower concentrations, individual neurons can use large number of peptide messengers, and the chemical heterogeneity of peptides is much higher than classical transmitters.

A single neuron contains thousands of different compounds; as adjacent cells have differences in signaling molecules, pooled samples are even more complex. Collecting starting material is often problematic, time consuming and expensive and so isolating peptides from *Aplysia* involves mass-limited assays. Purifying new peptides requires multiple stages of a high efficiency separation followed by a detection scheme that provides extensive qualitative information while maintaining excellent sensitivity. Down scaling LC separations from conventional 4.6 to 0.5 mm diameter columns greatly increases the separation efficiency and results in more concentrated peaks, which are needed for this type of sample [7–11]. With the advent of micro-sequencing peptide information can be obtained from as little as 5 pmol of material [8], easily accommodated using microbore LC.

For isolating and identifying peptides, a time-honored method is LC with bioassay [12,13]. For example, small cardioexcitatory peptide (SCP) was isolated from *Aplysia* using a bioassay of its effect on heart beat amplitude and heart rate [12]. Although bioassays can have excellent sensitivity, they tend to be time consuming and are difficult to adapt to the search for new peptides. The conventional detection techniques such as absorbance, electrochemical, and fluorescence detection are more universal, and therefore better suited to detect new peptides, although they are difficult to use alone because of the large number of peptides observed for even simple cellular samples. Hence, a large effort has been devoted to finding more information rich detection schemes such as mass spectrometry.

LC with electrospray MS is a quick and easy method of obtaining molecular masses of peptides eluting from the column [14–19]. As an alternative, LC with matrix-assisted laser desorption/ionization (MALDI)-MS detection offers easy off-line capabilities and similar performance to electrospray ionization, with the added benefit of fewer restrictions in solvents and buffers used for the LC separation. For example, salt removal can be achieved with chromatography and is used routinely in MALDI-MS sample preparation [20]. More common applications of off-line LC–MALDI-MS involve the analysis of protein digests to obtain a mass map for protein identification [21,22]. Recently, microbore LC in combination with MALDI-MS analysis has received more attention. Elicone et al. have reported using microbore LC to purify peptides for combined chemical sequencing and MALDI-MS analysis [23]. Another example utilizes a novel sample collection method, where the effluent from a capillary LC column was deposited on a poly-(vinylidene difluoride) (PVDF) membrane [24]. More recently, microcolumn LC was interfaced with MALDI-MS to separate peptides present in single neurons [25]. One additional advantage of using microbore LC in conjunction with MALDI-MS detection is that only a very small amount of sample is consumed for each assay, leaving almost an entire LC fraction available for further chemical or biological characterization or additional stages of separation.

MALDI-MS can be used to directly profile the peptides from single cells and neuronal processes by simply placing the cell or tissue on the target in the appropriate matrix [26–28]. While this method provides a simple and sensitive way of determining species present, it is difficult to fully characterize peptides in such complex matrices. Although direct MALDI-MS of cells has been used for a number of studies, the ability of direct MALDI-MS to detect most peptides present in a cell has not been evaluated. We compare direct MALDI-MS of *Aplysia* connectives to the peptides detected by pooling connectives and isolating with LC prior to MALDI-MS analysis. We present the strategy of using single cell MALDI-MS to find novel peptides based on M_r, and then LC with MALDI-MS guided purification in order to isolate the peptides for sequencing and further study. Specifically, we report using MALDI-MS to rapidly screen for peptides in the *Aplysia* pleural abdominal connective (PAC) and then use MALDI-MS to guide multiple stages of LC purification to isolate the unknown peaks for sequencing analysis.

2. Experimental

2.1. Animals

Aplysia californica weighing 100–200 g were obtained from *Aplysia* Research Facility (Miami, FL, USA), while those weighing 200–350 g were purchased from both Pacific Biomarine (Venice, CA, USA) and Marinus (Long Beach, CA, USA). Animals were maintained in artificial sea water (Instant Ocean, Aquarium Systems, Mentor, OH, USA) at 14°C.

2.2. Connective and cellular sample preparation

Ganglia with intact connectives and commissures were removed after an injection of 390 mM $MgCl_2$ equal to one-half of each animal's body weight. In some cases a moderate protease treatment (e.g., 1% Protease Type IX for 30–60 min at 34°C) was used to soften the connective tissues prior to desheathing. Salts were eliminated by a previously described approach [26]. Briefly, interganglionic connectives were cut and removed, and the physiological saline was replaced with an aqueous MALDI matrix solution, 10 mg/ml of 2,5-dihydroxybenzoic acid (DHB; ICN Pharmaceuticals, Costa Mesa, CA, USA) in ultrapure water (Millipore, Bedford, MA, USA). Tungsten needles were used to desheath and isolate small (<1 mm) sections from the interior of the nerve. Each section was then placed onto a MALDI-MS sample plate containing 0.5 μl of matrix solution. After drying at ambient temperature, samples were either frozen or analyzed immediately. To generate Table 1, 83 samples were obtained from eight animals.

2.3. Microbore LC of homogenates

PACs from 25 animals were pooled for microbore LC separation. The connectives were collected on dry-ice and subsequently stored at −80°C. Samples were homogenized in a micro-homogenizer (Jencons Scientific, UK), sonicated (Branson 2200, Danbury, CT, USA), and centrifuged (Baxter, McGaw Park, IL, USA). The supernatant was removed, freeze-dried (Labconco, Fisher Scientific, Itasca, IL, USA), and resuspended in 50 μl of 2% acetonitrile in 0.1% trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA). A volume of 20 μl of the extract was injected in a reversed-phase microbore LC instrument (Magic 2002, Michrom BioResources, Auburn, CA, USA) consisting of a Reliasil C_{18} column (150×1.0 mm) with 300 Å packing. The flow-rate was 25 μl/min at ambient temperature. The column was equilibrated with solvent A, and a gradient was developed from 0 to 80% of solvent B in 30 min and then 80–98% of solvent B in 10 min. Samples were collected by a fraction collector (Gilson FC 203B, Middletown, WI, USA), and each fraction was screened by MALDI-MS; 0.5 μl of each LC fraction was deposited onto a MALDI-MS sample plate followed by the same volume of DHB matrix solution. Unless otherwise specified all solvents were purchased from Fisher Scientific and were reagent quality or better. LC solvents were selected and adjusted to optimize separation according to procedures outlined below.

2.4. MALDI-MS

Mass spectra were obtained using a Voyager Elite mass spectrometer equipped with delayed ion extraction (PerSeptive Biosystems, Framingham, MA, USA). A pulsed nitrogen laser (337 nm) was used as the desorption/ionization source, and positive-ion mass spectra were acquired using reflectron mode. Each representative mass spectrum shown is the unsmoothed average of 64–128 laser pulses. Mass calibration was performed externally using either bovine insulin (Sigma, St. Louis, MO, USA) or a previously calibrated spectrum obtained from *Aplysia* bag cells. The average mass assignment error is 190 ppm, which is typical for the cellular peptide assay using external calibration [29].

2.5. Database searches

The peptide sequence was searched using the FASTA protocol [30] against the SwissProt database [31] compiled by the European Molecular Biology Laboratory.

Table 1
Peptides detected in pleural abdominal connectives (PAC) with MALDI-MS and LC–MALDI-MS

Average $[M+H]^{+}_{obsvd}$ (a)[a]	Peptide identity	Direct MALDI-MS	LC–MALDI-MS
599.38	FMRFamide	x[b]	x
766.41	FRF_A		x
769.35	FPF_C		x
788.46	MM-D		x
838.58	MM-H		x
846.45	MM-A		x
861.55	MM-C		x
1009.4	α-BCP [1–8]		x
1113.5	Buc N	x	x
1122.8	α-BCP [1–9]	x	x
1125.9	NPY [32–40]	x[b]	x
1141.8	SCP_B	x	x
1152.6	Buc_B	x	x
1163.2	Sensorin A	x	x
1169.5	Buc C	x	x
1200.5	LUQIN	x	x
1278.0	SCP_A	x	x
1314.9	CP1	x	x
1362.9	pG1u-R3–14 II		x
1381.3	R3–14 II		x
1539.2	Pedal peptide	x	x
1549.5	C4 of CP1 precursor	x	x
1563.1	$ProELH_{188–203}$		x
1575.2	ELH [1–14]	x	
1863.2	ϵ-BCP	x	x
2215.6	$AP_{9–27}$	x	x
2626.1	C2 of CP1 precursor	x	x
2828.5	ELH [15–36]	x	
2860.1	R15β		x
2899.9	pGlu-R3–14 I	x	x
2917.8	R3–14 I	x	x
2961.3	AP	x	x
3092.0	Unknown	x	x
4385.4	ELH	x	x
4409.3	δ-BCP		x
4477.2	C5-RR-CTP	x	x
4481.5	pGlu-R15γ	x	x
4592.5	CP2	x	x
4688.1	NPY		x
4926.0	HRBP		x
5014/5022	Unknown	x	x

FMRFamide, phe-met-arg-phe-amide; FRF_A, phe-arg-phe-amide; MM, myomodulin; Buc, buccalin; BCP, bag cell peptide; SCP, small cardioactive peptide; ELH, egg-laying hormone; NPY, neuropeptide Y; CP1, cerebral peptide 1; CP2, cerebral peptide 2; C2, C3, C4, connecting peptides 2, 3, 4 of APGWamide precursor/CP1 precursor; C5-RR-CTP, connecting peptide 5 and C-terminal peptide with double Arg residues uncleaved; HRBP, histidine-rich basic peptide.

x in the table indicates detection of a peak; blank indicates that the peak was not detected.

[a]The masses below 2000 are listed as monoisotopic protonated molecular masses; the masses above 2000 are listed as average molecular masses.

[b]Peptides were detected in less than 10% of PAC samples due to the interference of lipids from cellular matrices.

3. Results and discussion

As peptides transported between major ganglia are likely to have important biological function, we recently reported on using MALDI-MS to directly profile the peptides in different connective nerves [29]. In particular, the pleural abdominal connective (PAC) is the longest connective within the CNS of *Aplysia*, and so easiest to completely characterize. A large number of previously characterized (based on M_r) as well as many unknown peptides were detected in PACs. In the current study, we use microbore LC to isolate and purify several novel peptides from the complex mixture contained within the PACs for further characterization. We report here a strategy to combine the speed and ease of MALDI-MS analysis with LC microseparation to allow novel peptides to be isolated, purified and sequenced.

3.1. Direct MALDI-MS analysis of PAC

A typical LC separation yields many different peaks representing a wide array of species; therefore the ability to choose peaks of interest for further analysis is desired. Direct MALDI-MS-based profiling of neurons and nerves allows the determination of many of the peptides present within the sample. As shown in Fig. 1, MALDI-MS of a sub-section of a PAC reveals the presence of several previously characterized peptides including buccalin, cerebral peptides, α-bag cell peptide, acidic peptide (AP) and egg-laying hormone (ELH) and multiple AP and ELH novel processing forms [28]. Table 1 lists the major peaks observed in PACs based on 83 samples with a mass listed as detected if a peak is detected in more than 10% of the samples, unless otherwise noted. Interestingly, many unassigned molecular masses (unknown peptides) are observed in the spectra. In particular, two peaks paired together at 5014 and 5022 are detected in many major connectives including the PAC and cerebro-pleural connective [29]. In an effort to further characterize these two novel peptides, a microbore LC is used to isolate and purify them from the complex cellular matrices.

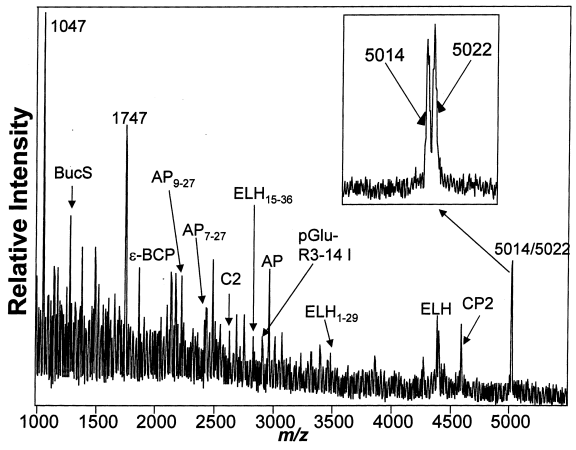

Fig. 1. MALDI-MS spectrum obtained from a section of pleuro-abdominal connective (PAC) placed directly on the MALDI target. Peaks that match previously characterized *Aplysia* peptides are labeled with the peptide name.

3.2. Optimization of extraction and separation

Because these biological samples contain many different peptides in varying amounts and the difficulty associated with softening the connective tissue, this analysis requires an extraction method which can extract as much of the species as possible. Fig. 2 describes the extraction protocol utilized in this work. We verified the procedure by testing whether the novel and uncharacterized peptide with a monoprotonated molecular mass of 5014 was efficiently extracted. This verification was performed by extracting the material from the nerves twice, injecting the aliquots onto the column and after drying/resuspension, measuring spectra on the LC fractions. The second extraction yielded a chromatogram void of peptides. In addition, no peaks are observed with direct MALDI-MS assay on the remaining tissue, further verifying that the peptides are efficiently extracted.

3.3. Comparison of direct and LC–MALDI-MS of PAC

Although direct MALDI-MS provides a relatively complete peptide profile at the M_r range of 1000–7000, it has difficulty in identifying the peaks in the low-M_r region ranging from 500 to 800 due to the interference from phospholipids present in tissue samples. Table 1 compares peptides detected in a PAC using both direct MALDI-MS and LC–MALDI-MS. This table illustrates that some peptides

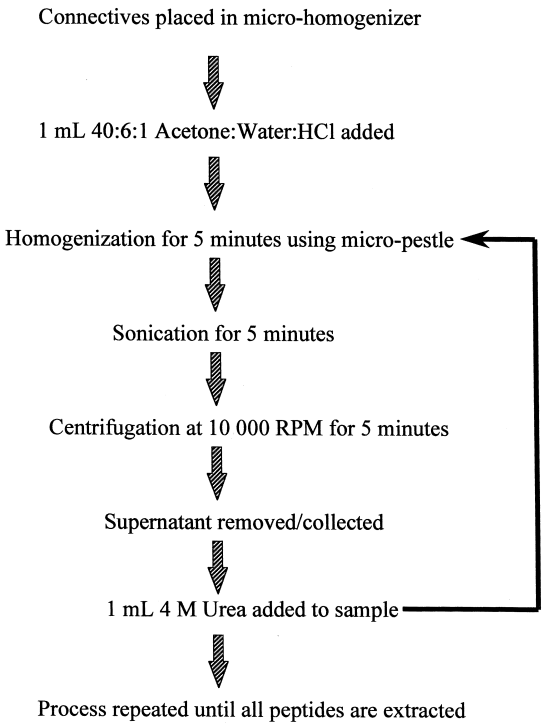

Fig. 2. Experimental procedure utilized to extract peptides from connectives prior to injection onto LC.

are detected with MALDI-MS alone while others are only seen following LC separation.

Obviously, direct MALDI-MS is a quicker and simpler procedure. However, LC–MALDI-MS offers a more complete peptide profile than direct MALDI-MS, in that it detects essentially all the peaks that are seen in PAC tissue sample by direct MALDI-MS. Many low-M_r peptides, such as myomodulins, FRF_A and FRF_C [32], were not detected by direct MALDI-MS, and FMRFamide and NPY fragment were only observed in less than 10% of the PAC samples. This is due to many intense peaks, which occupy the same mass range, that we assign to phospholipids from cellular matrices. In contrast, LC–MALDI-MS eliminates the problem by separating out the cellular components, such as lipids, so that low-M_r peptides are readily analyzed in a LC fraction. In addition, LC–MALDI-MS also alleviates the problem of analyte suppression [33,34] which is encountered in the direct MALDI-MS analysis when a species of interest is present in much lower amount than the major components. One disadvantage is that peptides close to the detection limit of direct MALDI-MS are sometimes not observed (for example, ELH fragments) likely due to dilution losses during LC purification. By using MALDI-MS to screen for LC fractions to keep track of preselected peptides, isolation and purification can be guided to allow the separation of closely related species for further sequencing. Lastly, a major advantage of LC–MALDI-MS is that it only uses a small portion of the LC fraction, allowing further chemical and/or biological characterization.

3.4. LC isolation and MALDI-MS analysis of 5014/5022

Two unknown molecular masses, 5014 and 5022, are observed in the PAC and are often the most intense peaks observed in the spectra using direct MALDI-MS. They are seen in highest intensity at the end of the PAC near the bag cell neurons. Since these compounds are a higher molecular mass than many neuropeptides, isolation of at least 100 pmol is ideal for sequencing. Therefore, connectives were pooled from 25 animals, extracted, freeze-dried, and injected onto the LC column and subsequent fractions were collected. Fig. 3 is a series of chromatograms involved at each stage of the three separations in an attempt to isolate 5014 and 5022 in separate fractions. The first chromatogram is indicative of crude sample injections, and contains many different species with the peak of interest being smaller than several other peaks. MALDI-MS is used to identify which fraction contains the peptides of interest. Fig. 4A shows the mass spectrum from the appropriate fraction marked in the initial separation (Fig. 3A). Several peaks are present including those that we are interested in isolating. This fraction is subsequently freeze-dried, resuspended in 20 μl of aqueous acetonitrile, and injected onto the LC system. In this case the solvents are changed to aid in the isolation of a pure peak. Fig. 4B shows the MALDI-MS spectrum of the appropriate isolated fractions (Fig. 3B) from the second stage separation illustrating that now both 5014 and 5022 are contained in the same fraction with almost all of the other components absent. For the final separation (Fig. 3C) of this isolated fraction, a different counter-ion, namely

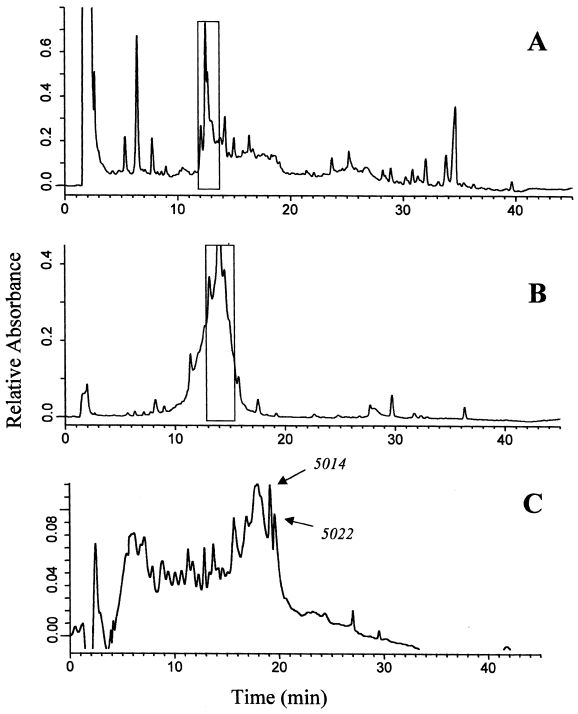

Fig. 3. Series of LC chromatograms illustrating the three stages of separation from crude sample to purified fraction. For separation (A): solvent A, acetonitrile–water+0.1% TFA (2:98); solvent B, acetonitrile–water+0.09% TFA (95:5). For separation (B): solvent A, [isopropanol–methanol–acetonitrile (1:1:2)]–water+0.1% TFA (2:98); solvent B, [isopropanol–methanol–acetonitrile (1:1:2)]–water+0.09% TFA (95:5). For separation (C): solvent A, acetonitrile–water+0.1% HFBA (2:98); solvent B, acetonitrile–water+0.09% HFBA (95:5).

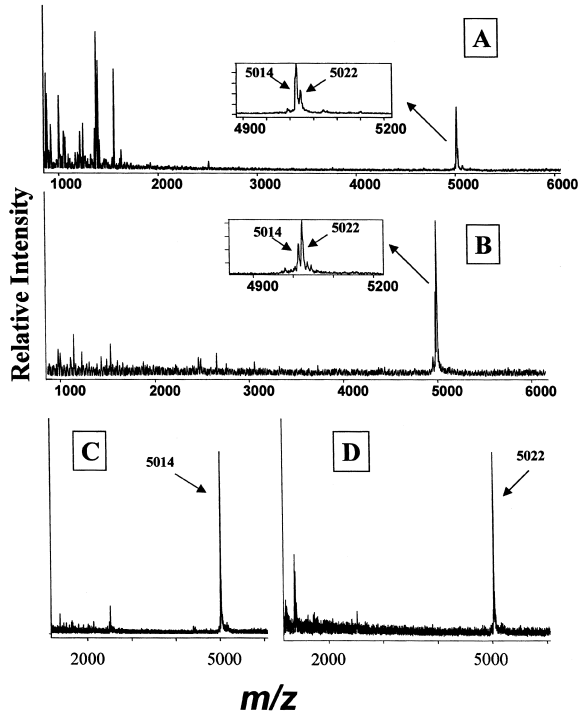

Fig. 4. MALDI-MS spectra of individual fractions containing 5014/5022 at each stage of the separation shown in Fig. 3.

HFBA instead of TFA is used, resulting in the separation of 5014 and 5022 into different fractions (Fig. 4C and Fig. 4D, respectively).

3.5. Characterizing ACa/ACb

Isolation of approximately 150 pmol of either species is enough to allow sequencing. Only a partial sequence is required to perform further analysis using molecular biological approaches to identify the appropriate gene. These two peptides were N-terminally blocked, so that no sequence was obtained with N-terminal Edman sequencing. A partial sequence was obtained after heating with CNBr to cleave off the blocking residue(s). The N-terminal sequencing analysis via Edman degradation revealed a partial sequence of: $P_{(OH)}$GPHGXZGI$P_{(OH)}$GVVG (with X indicating unassignable residue). The hydroxyproline, $P_{(OH)}$, and glycine (at every third residue) is indicative of a collagen-like structure, confirmed by searching this sequence against the SwissProt database; hence, this peptide is given the name *Aplysia* collagen a (ACa). Sequencing analysis of 5022 revealed a blocked peptide with the same hydroxyproline and glycine pattern of a collagen (ACb). These two are the first collagens to be partially characterized in *Aplysia*, although the known sequence represents only 24% of the total peptide. Further work is involved with a complete characterization of these two peptides.

Collagen is a term for many different types of closely related proteins, usually taking the form of fibers with high tensile strength all containing hydroxyproline (instead of proline) and glycine at every third residue. They are utilized mainly as structural supports to cells and tissues in both vertebrates and invertebrates. Interestingly, in mammals, some col-

lagens interact with peptide hormones, such as angiotensin and insulin [35–38]. Collagen may also play a major role in the development of diabetes and other age-related pathologies [39]. Furthermore, some monoamines, such as serotonin, noradrenaline, and adrenaline, as well as the cytokine interleukin I, are released in a collagen-induced manner [40,41].

In addition, recently it has been suggested that collagen may also play a role in learning and memory formation of *Aplysia* [42]. In this study, Liu et al. provide evidence that tolloid/human bone morphogene protein 1 (BMP 1)-like molecules play a role in the processing of procollagens. Furthermore, they show that this tolloid/BMP 1-like protein may be involved in the regulation of the morphology and efficacy of synaptic connections through the modification of the cytoskeleton structure in the growth process within the sensory neuron. These changes are associated with long-term sensitization, resulting in the formation of long-term memory.

ACa and ACb have been observed in major connectives and several cerebral cellular clusters, and have structural characteristics of collagen. We are studying whether these peptides are involved primarily in structural support or if they interact with neuropeptides within the *Aplysia* CNS.

4. Conclusions

As samples become more difficult to analyze due to their small volumes and complexity, a technique that can separate and detect minor species is required. Microbore and capillary LC allows relatively small amounts of material to be isolated and the addition of MALDI-MS provides needed qualitative information. A direct comparison of MALDI-MS and LC–MALDI-MS reveals that direct MALDI-MS is effective at assaying most peptides from cells and nerves except at low molecular masses. The combination of LC with MALDI-MS guided purification allows novel peptides to be isolated from complex cellular samples and subsequently used for amino acid sequencing. Using peak tracking, the isolation of previously unreported peptides such as ACa and ACb presented here becomes easier.

Acknowledgements

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