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Review

Molluscan attractins, a family of water-borne protein pheromones with interspecific attractiveness

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Abstract

The marine mollusk *Aplysia* releases the water-borne pheromone attractin during egg laying. This small protein stimulates the formation and maintenance of mating and egg-laying aggregations. Attractin has been characterized from five *Aplysia* species: *A. californica*, *A. brasiliana*, *A. fasciata*, *A. vaccaria*, and *A. depilans*. We describe here the isolation of attractin from *Bursatella leachii*, and show that it belongs to the same protein family. The pattern of residue conservation, especially the six invariant cysteines, suggests that all of these attractins have a common fold. The nuclear magnetic resonance solution structure of *A. californica* attractin contains two antiparallel α-helices, the second of which contains the heptapeptide sequence IEECKTS that has been implicated in attractin function. Synthetic peptides containing this IEECKTS region are attractive, and mutating surface exposed charged residues within this region of attractin abolishes attractin activity. This suggests that the second helix is an essential part of the receptor-binding interface. In contrast to the peptide pheromonal attractants in amphibians, which are species specific, the attractins are, to our knowledge, the first water-borne peptide or protein pheromone family in invertebrates and vertebrates that are not species specific.
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Keywords: Albumen gland; *Aplysia*; Attractin; *Bursatella leachii*; Mollusk; Protein pheromone

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1. Introduction

Exchange of pheromones and small signaling molecules between organisms is the most ancient form of communication. Pheromone-based signaling systems have been described for ciliated protozoans [29], yeast [23], insects [31,44,46], mollusks [9,10,13,38,52,61,62], worms [43,63], fish [25], amphibians [21,45,55], rodents [34,49] and humans [47]. Many of these systems rely on the binding of small volatile molecules, such as aliphatic carboxylic acids or alcohols, farnesenes, and N- and S-containing unsaturated ring compounds [8,34] to pheromone binding proteins such as lipocalin family members [26] and aphrodisin [54]. However, four water-borne peptide/protein pheromones have been characterized in invertebrates by both chemical and behavioral methods in intact animals: the protein pheromones attractin, enticin, and temptin from the marine opisthobranch mollusk *Aplysia californica* [13–16,37–39,48,52] and the peptide pheromone nereithione from the marine polychaete worm *Nereis* [43,63]. Peptide pheromones from the protozoan *Euplotes raikovi* (E*r* pheromones; [27]) have been structurally characterized and have structural similarities to attractin, as we will see later in this review.

A. californica is a simultaneous hermaphrodite that does not normally fertilize its own eggs. Field studies [4,24,50,51] have shown that *A. californica* are solitary animals most of the year, but during the reproductive season, they move into breeding aggregations, mate and lay eggs. The aggregations usually occur where egg cordons are laid, often deposited one on top of another. Most of the egg-laying animals mate simultaneously as females even though mating does not cause reflex ovulation [6], suggesting that egg laying precedes mating in the aggregation and that egg laying may release pheromones that establish and maintain the aggregate.

Similar observations have been made in the lab when *Aplysia* were not individually caged [4,6,50,51] and behavioral studies have shown that egg-laying animals with cordons are more attractive than sexually mature, but non-laying conspecifics [2,3,19,40]. T-maze assays show that at least some of the attractants originate from the egg cordon and are water-borne, since: (i) recent egg-layers without egg cordons are no more attractive than non-laying conspecifics; (ii) recently deposited egg cordons are attractive, with or without a non-laying conspecific; and (iii) both recently deposited egg cordons and their eluates increase the attractiveness of non-laying conspecifics following their addition to bathing seawater [35,36].

After ovulation, eggs travel to the fertilization chamber which is surrounded by the albumen gland, a large exocrine organ (Fig. 1) [12,41]. This gland packages eggs into a long string-like cordon, which has a high surface-to-volume ratio. Egg cordons are considered to be a source of both water-borne and contact pheromones, which attract animals to the area and induce them to mate and lay eggs [5,36]. Pheromones play a significant role in coordinating reproductive activity in *Aplysia*. Of the pheromones produced by the albumen gland, attractin has been extensively characterized; the behavioral repertoire of enticin and temptin in intact animals has been less studied [13].

We have isolated attractins from five *Aplysia* species and shown that they fall into two distinct sequence groups [39]. We report here the characterization of attractin from *Bursatella leachii* (Burle attractin), which is only 21% identical to the other attractins but shares certain highly conserved

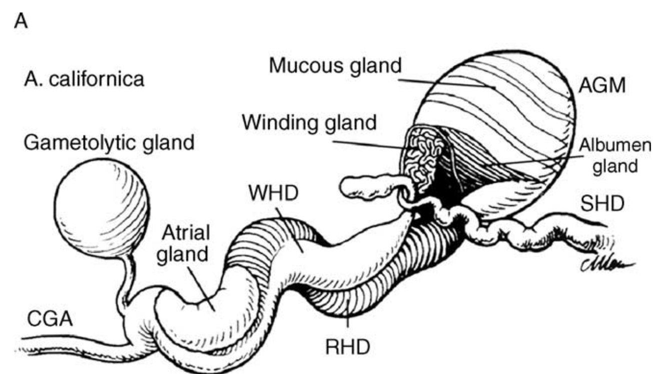

Fig. 1. Schematic diagram of the reproductive tract of *Aplysia californica*, which is representative of most of the *Aplysia* species examined (modified from [41]). Following ovulation, eggs are transported from the ovotestis (which would be at the far right) through the small hermaphroditic duct (SHD) to the accessory genital mass (AGM), where they are fertilized and packaged into a cordon. Packaging occurs as the eggs are transported past (or through) the three exocrine components of the AGM: the mucous, winding and albumen glands. The albumen gland makes up the bulk of the structure. The accessory genital mass is connected to the common genital aperture (CGA; far left) by the large hermaphroditic duct, which is composed of the red hemiduct (RHD; the oviduct) and the white hemiduct (WHD; the copulatory duct). The exocrine atrial gland is associated with the oviduct and secretes into the large hermaphroditic duct.

amino acids. Sequence alignments of these proteins were used to identify conserved residues likely to play a role in attractin function. These residues mapped to discrete areas on the 3D structure of *A. californica* attactin (Aplca attractin) [16]. Here, we summarize these results and review the structural, functional, and behavioral aspects of the molluscan attractin family.

2. Materials and methods

2.1. Purification and characterization of Bursatella leachii attractin

Albumen glands were removed from *B. leachii*, frozen on dry ice, and subsequently extracted at 4 °C in 0.1% heptafluorobutyric acid (HFBA) using a Polytron homogenizer and sonicated. The extract was centrifuged for 20 min at 48,000 × *g* (4 °C) and the supernatant was purified on C18 Sep-Pak Vac cartridges (Waters Corp.). Peptides and small proteins were eluted with 50% acetonitrile (CH_3CN)/0.1% HFBA and lyophilized. The lyophilizate was resuspended in 0.1% HFBA and applied to a semi-preparative Vydac C18 RP-HPLC column (10 mm × 250 mm). The column was eluted with a two-step linear gradient of 0.1% HFBA and 100% CH_3CN/0.1% HFBA (0–10% CH_3CN/0.1% HFBA in 5 min; 10–34% CH_3CN/0.1% HFBA in 85 min). The column eluate was monitored by absorbance at 215 nm, and 1-min (1.75-ml) fractions were collected. For chemical characterization studies, fractions containing attractin were pooled, lyophilized, reduced with 2-mercaptoethanol, alkylated with 4-vinylpyridine, and purified on an analytical Vydac C18 RP-HPLC column (4.6 mm × 250 mm), and 1-min (1-ml) fractions were collected. The same gradient conditions were used, except that 0.1% trifluoroacetic acid was the counterion. The purified fraction was characterized by sequence analysis and used for trypsin digestion.

Burle attractin residues 54 and 55 were determined by 3′-rapid amplification of cDNA ends (3′-RACE). 3′-RACE primer sequences are:

OL1, 5′-ATCGATGGTCGACGCATGCGGATCCAAAGCTTGAATTCGAGCTCT$_{17}$-3′; OL2, 5′-AAY(Y = C + T) W(W = T + A)S(S = G + C)N(N = A + T + C + G)GAYGAYCCNTGYG AYGA-3′; OL3, 5′-ATCGATGGTCGACGCATGCGGATCC-3′; OL4, 5′-ACNGAYCAYGAR(R = A + G) GARTGYATGGC-3′; OL5, 5′-GCGGATCCAAAGCTTGAATTCGAG CTCT-3′.

Total RNA was isolated by TRIzol extraction [11] of *B. leachii* albumen glands, and first-strand cDNA was generated by reverse transcription of total RNA using an antisense adaptor primer OL1 and the Superscript Preamplification System for First Strand Synthesis (Invitrogen). PCR was performed using a degenerate sense primer corresponding to the N-terminus of Burle attractin (OL2) and an antisense primer (OL3). Samples were heated at 94 °C for 2 min and amplified for 45 cycles (94 °C, 25 s; 35 °C, 25 s; 72 °C, 1 min), followed by a 7-min extension at 72 °C. The primary PCR (1 μl) was reamplified using a nested degenerate sense primer corresponding to Burle attractin residues 32–39 (OL4) and the semi-nested antisense primer (OL5). 3′-RACE products were cloned by insertion into pCR4-TOPO (Invitrogen) and nucleotide sequence analyses were performed.

3. Results

3.1. Purification and sequence determination of Bursatella attractin

Burle attractin was purified to homogeneity by using two sequential RP-HPLC gradients (data not shown). *B. leachii* albumen glands contained a unique and abundant attractin, and 3′-RACE identified Thr-54 and Thr-55 in Burle attractin (Table 1). Sequence analysis of three independent *B. leachii* 3′-RACE clones predicted a C-terminal Arg-60 residue followed by a stop codon; Arg-60 was not detected by Edman sequence analysis.

4. Discussion

4.1. Structure of attractins

Microsequence and 3′-RACE analyses have revealed a single distinct attractin-related protein in albumen gland extracts

Table 1
Chemical sequence analysis of *Bursatella* attractin

	Fragment	Sequence	Procedures
Reduction and	1–60	NSD*PXDELVLQENXDIEVQQXQESGGQDXSTDHEEXMARLRLSXDDSNVDFE--A-P	Edman
alkylation		NSDDP	Edman
Reduction and	1–60	LSXDDSNVDFE--ASPS	Edman
alkylation		ARLRLSCDDSNVDFETTASPSR	DNA sequence
Reduction and	43–60	NSDDPCDELVLQENCDIEVQQCQESGGQDCSTDHEECMARLRLSCDDSNVDFETTASPSR	
alkylation/trypsin			
3′-RACE/cloning	39–60		
Sequence	1–60		

X, pyridylethyl cysteine; Edman, Edman degradation sequencing; 3′-RACE, rapid amplification of cDNA ends. Asterisk (*) indicates microsequencer malfunction at this residue.

Table 2
Subgenera and species in which Aplysia attractins have been characterized

Subgenus	Species
Pruvotaplysia	*parvula*, *punctata*
*Neoaplysia**	*californica**
*Varria**	*brasiliana**, *fasciata**, *cervina*, *cornigera*, *cronullae*, *dactylomela*, *denisoni*, *extraordinaria*, *gigantean*, *gracilis*, *inca*, *keraudreni*, *kurodai*, *maculata*, *morio*, *oculifera*, *pulmonica*, *rehderi*, *reticulata*, *robertsi*, *sagamiana*, *sowerbyi*, *syneyensis*, *willcoxi*, *winneba*
*Aplysia**	*depilans**, *vaccaria**, *cedrosensis*, *dura*, *juliana*, *nigra*
Phycophyla	*euchlora*

Subgenera and species that have been examined for attractins are indicated by asterisks. Modified from Kandel [20].

of *A. californica*, *A. brasiliana*, *A. fasciata*, *A. depilans*, *A. vaccaria*, and *Bursatella leachii* [38,39]. The five *Aplysia* attractins that have been characterized to date are representatives of three subgenera that comprise 32 of the 35 known *Aplysia* species and ~15% of all *Aplysia* species (Table 2). Aplca attractin, a 58-residue protein with three intramolecular disulfide bonds (C4–C41; C13–C33; C20–C26; Fig. 2) [38,48], was originally isolated from *A. californica*, a Pacific Coast species. The cDNA of Aplca attractin encodes a signal peptide and a 58-residue protein without a transmembrane domain [15]. The complete sequences of the six attractins are shown in Fig. 2. The six cysteines, three charged residues (Asp-5, Asp/Glu-22, and Glu-39), and the sequence Ile^{30}-Glu^{31}-Glu^{32}-Cys^{33}-Lys^{34}-Thr^{35}-Ser^{36} (IEECKTS) are conserved in all five *Aplysia* attractins; the six cysteines, Asp-5, Glu-22, Glu-31, and Glu-32 are also conserved in Burle attractin (*Aplysia* residue numbers).

On the basis of their sequence similarity with Aplca attractin, the attractin proteins fall into three groups: Aplca attractin, *A. brasiliana* (Aplbr attractin), and *A. fasciata* (Aplfa attractin; 91–95% amino acid sequence identity); *A. depilans* (Aplde attractin) and *A. vaccaria* (Aplva attractin; 41–43% amino acid sequence identity); and Burle attractin (21% amino acid sequence identity). *A. brasiliana* and *A. fasciata* inhabit different geographical areas, and *A. depilans* and *A. vaccaria* also inhabit different geographical areas.

As would be expected on the basis of their near identity, Aplca attractin attracts *A. brasiliana*, a species found in the Gulf of Mexico. Aplbr attractin differs from Aplca attractin at only three amino acids (Fig. 2), is deposited on egg cordons, and elutes into the seawater following deposition [39]. We thus used *A. brasiliana* in all of our attractin bioassays, since *A. californica* locomote slowly, or not at all, during T-maze attraction assays, and tend to crawl out of T-mazes before being exposed to chemical test stimuli. In marked contrast, *A. brasiliana* are rapid swimmers that can reach test stimuli in T-maze assays in less than ~15 s. We have also used *A. brasiliana* to bioassay Aplva attractin in T-maze assays; Aplva attractin is an example of the second group of attractin types.

Four of the *Aplysia* attractins are posttranslationally modified in the N-terminal region (Asn-8, Aplca, Aplbr, Aplfa; Asn-25, Aplde) and two in the C-terminal region (Ser-49, Ser-50, Thr-51, and Thr-52, Aplva; Ser-49 and Ser-50, Aplde). Mass spectrometry showed that native Aplca attractin is 21 wt.% carbohydrate as the result of *N*-linked glycosylation at Asn-8 [38]. However, non-glycosylated recombinant attractin is significantly active in T-maze and mating assays, demonstrating that *N*-glycosylation of Aplca attractin is not required for attraction [38,39,48]. Since Aplca attractin is progressively degraded from the C-terminus but not from the N-terminus (Fig. 3), and only the N-terminal 41–47 amino acids are required for attraction activity [38], posttranslational modifications in the C-terminal region of Aplva attractin and Aplde attractin may serve to prolong their half-life and biological activity or to serve some other unknown function, but this has not been examined. Mass spectra of Aplva attractin are consistent with a glycosylated protein with variable numbers of hexose units; Aplde attractin spectra also suggest significant posttranslational modification [39].

4.2. 3D structural basis of Aplysia attraction

Recombinant Aplca attractin expressed in insect cells was shown to be attractive in the T-maze assay [37,48]. We were intrigued by some similarities at the sequence level between the attractins and a family of pheromones isolated from *Euplotes raikovi*. This organism produces

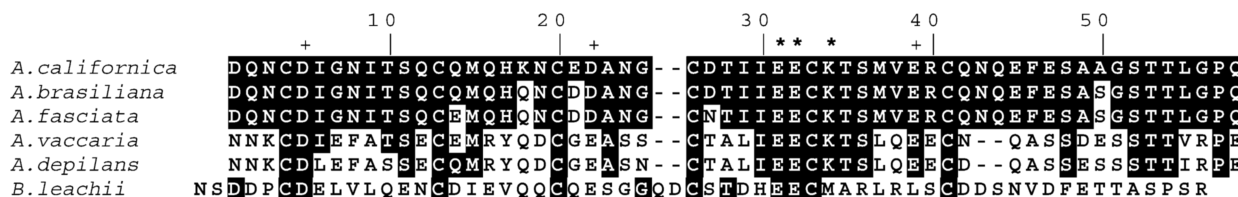

Fig. 2. Comparison of attractin sequences from *Aplysia californica* (accession no. A59060), *A. brasiliana* (B59060), *A. fasciata* (A59447), *A. vaccaria* (A59424), *A. depilans* (A59446), and *Bursatella leachii* (A59453). Identities are shaded black. *Asterisks* indicate amino acids (Glu-31, Glu-32, and Lys-34) substituted in one Aplca attractin triple mutant; *plus signs* indicate amino acids (Asp-5, Asp-22, and Glu-39) substituted in a second Aplca attractin triple mutant.

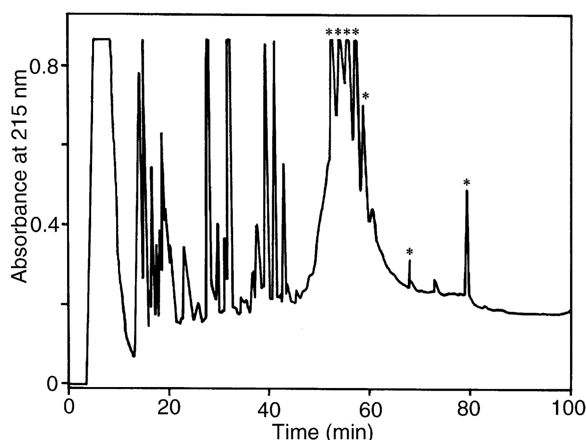

Fig. 3. Attractin is secreted during egg laying and degraded from the C-terminus. Egg laying was induced in *Aplysia californica*, and eluates were purified and concentrated on C18 Sep-Pak Vac columns and fractionated by C18 RP-HPLC. The seven peaks indicated by an asterisk contained the same N-terminal sequence, were breakdown products of Aplca attractin, and differed only in length.

several peptides (e.g., E*r*-1, E*r*-2, E*r*-10, E*r*-11) that differ greatly in their mature primary sequence, but retain the same compact three-dimensional (3D) structure consisting of 3 α-helices stabilized by three disulfide bonds [30,56]. However, we found that the disulfide bonding pattern of recombinant Aplca attractin (determined by proteolysis and mass spectroscopy of fragments) differed from that conserved in the E*r* family. The circular dichroism spectrum indicated some helical structure in attractin.

We thus decided to determine the solution structure of the unlabeled recombinant Aplca protein with nuclear magnetic resonance (NMR) [16]. We used homonuclear 2D TOCSY and NOESY spectra to obtain the sequential assignment. We used our in-house structure calculation suite NOAH/DIAMOD/FANTOM to determine the 3D structure and the final assignments of the NOESY spectrum simultaneously, applying our previous strategy using NOAH/DIAMOD to solve NMR solution structures [57–59]. The bundle of structures determined from the data is shown in Fig. 4A, the location of the three disulfide bonds is shown in Fig. 4B, and the ribbon structure with the amino acids conserved in *Aplysia* attractins is shown in Fig. 4C. A movie showing the 3D NMR structure of Aplca attractin can be found in the online Supplementary Information section (Movie 1). We found that the overall structure of attractin differed from the well-studied pheromones of the ciliated protozoan *Euplotes* in that the attractins have only two helices. However, the second helix of attractin, containing the IEECKTS motif, is quite similar to the third helix of E*r*-11 [16]. This third helix of the E*r* pheromones is involved in receptor recognition [28,56].

Based on sequence and structural data [16,48], we proposed that the heptapeptide sequence IEECKTS, conserved in attractins from five different *Aplysia* species, is indeed important for biological activity. This was tested in two ways. First, a synthetic constrained cyclic peptide that contains the conserved heptapeptide sequence is significantly attractive in T-maze bioassays [14]. Second, altering the three charged amino acids in the IEECKTS sequence (Glu-31, Glu-32, Lys-34; Fig. 2), which forms the second helix of the 3D structure, effectively abolishes the activity of attractin in T-maze assays [39]. In contrast, mutating three conserved charged residues at other areas of the peptide (Asp-5, Asp/Glu-22, Glu-39; Fig. 2) slightly reduces but does not destroy attractin activity [39]. All attractins characterized to date have six conserved cysteines and three acidic residues corresponding to Asp-5, Glu-31, and Glu-32 of Aplca attractin. These three residues are solvent exposed in the 3D NMR solution structure of Aplca attractin (Fig. 4C) [16]. The 3D structure allowed us to distinguish residues that are conserved for structural reasons (e.g., cysteines, the core residue Ile-30) from those whose surface exposure could reflect a role in receptor recognition (Asp-5, Ser-11, Met-15, Asp/Glu-22, Glu-31, Glu-32, Glu-39) [16]. The triple mutant Aplca attractin E31Q, E32Q, K34Q (Fig. 2) lacks activity in T-maze assays, suggesting that Glu-31, Glu-32, and Lys-34 may be involved in receptor binding and pheromonal attraction, and may account for the interspecific attraction activity of attractin.

The signaling system may be even more fundamental. As noted above [48], attractins have similar sequence and structural properties to those of *Euplotes*. Although *Euplotes* pheromone sequences differ significantly, sharing only six cysteines and an N-terminal aspartic acid, all have the same compact "pyramid" 3D structure of three α-helices [27,28,30,32,56]. The E*r*-1 pheromone can bind to the mammalian receptor for interleukin-2 [53], raising the possibility that water-borne pheromones may be ancestors of cytokines in higher organisms.

In most organisms, sex pheromones attract potential mates (e.g., [22]). If mate attraction were the sole function of attractin, one might expect that the pheromone would attract only conspecifics. However, our data demonstrate that attractin is a relatively promiscuous signal [39]. This finding suggests that the 3D structure of attractin [16], which is compact and has two antiparallel helices stabilized by disulfide bonds, has been conserved during evolution. Other additional functions, for example the reduced latency to mating and the stimulation of hermaphroditic mating [37], may prevent alteration of the attractin structure.

4.3. Behavioral effects of attractin

Aplca attractin is attractive to *A. brasiliana*, reduces the latency to mating and increases the time spent mating (Fig. 5). The protein also reduces the latency to hermaphroditic mating, doubles the number of pairs mating as hermaphrodites, and increases the duration of the activity (Fig. 6) [37]. These effects may result from attractin stimulating both animals to mate as males [37]. These findings are consistent with field observations in which multiple *Aplysia* species are often found in the same egg-laying and mating aggregations,

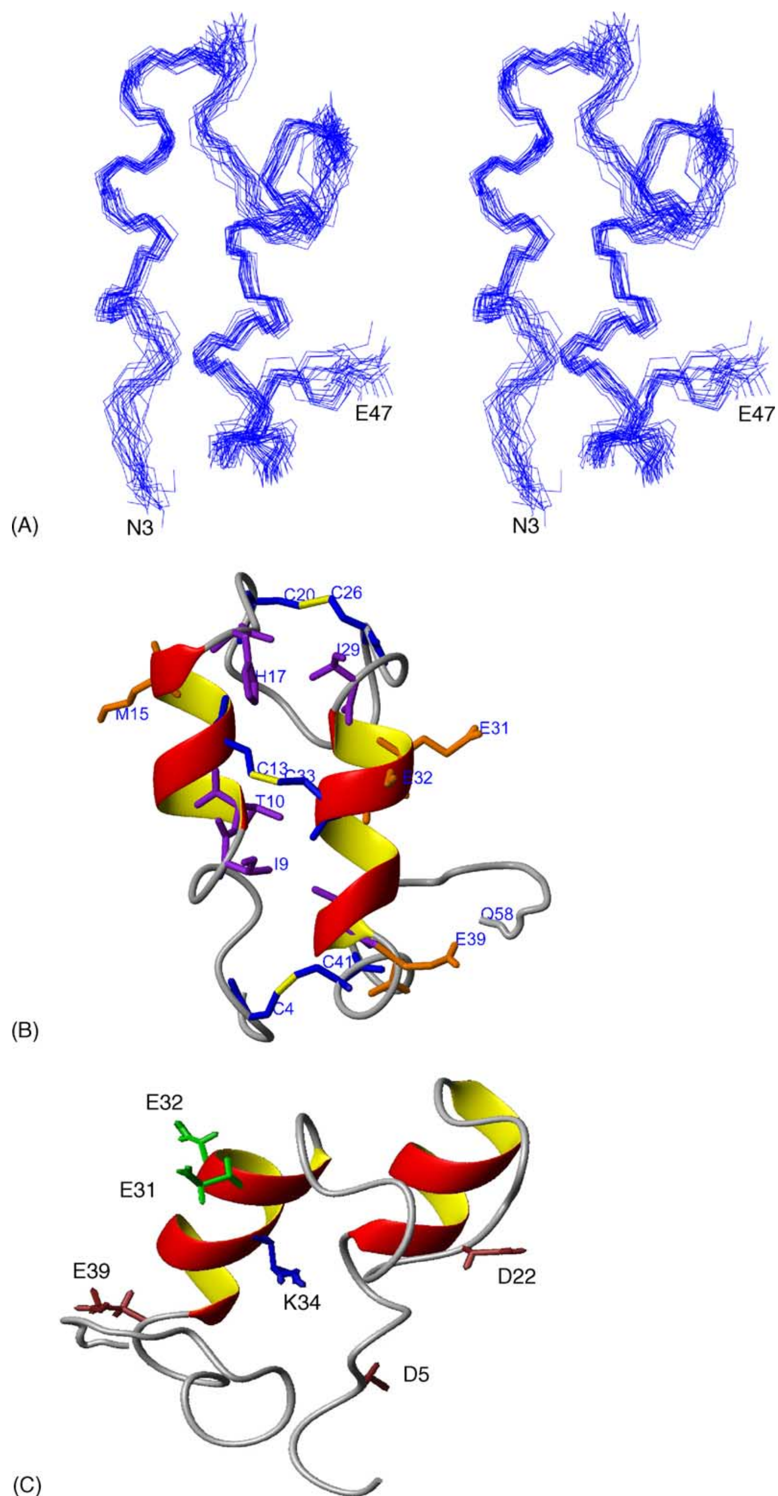

Fig. 4. NMR solution structure of Aplca attractin. (A) Bundle of 20 structures with the smallest target function from a NOAH/DIAMOD calculation (only the backbones are shown for residues 8–42). (B) Ribbon representation of one of the representative structures (that with the smallest root-mean-square-deviation from the mean) with the side chains of core residues (magenta) and conserved solvent exposed residues (orange). The disulfide bonds between cysteines (yellow) and the helical regions T10-Q16 and I30-V38 are also shown in this structure. (C) NMR structure showing the side chains of the residues altered for mutation studies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

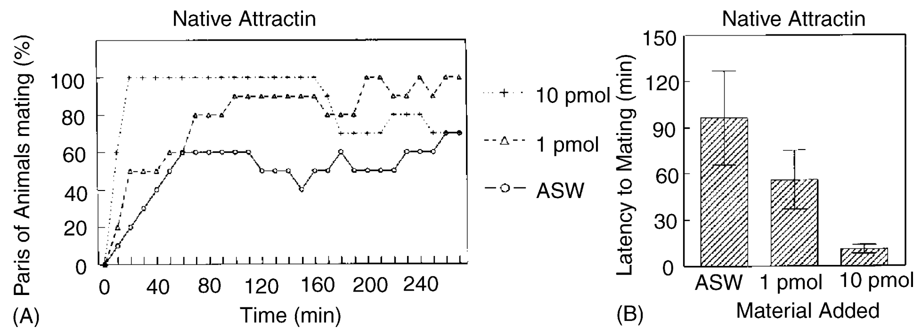

Fig. 5. Aplca attractin reduces the latency to mating in *Aplysia brasiliana*. (A) The percentage of animals mating at early time periods was increased when native Aplca attractin was placed in the adjacent seawater. (B) The latency to mating was reduced by placing either 1 pmol or 10 pmol native Aplca attractin in the seawater.

for example, *A. californica* and *A. vaccaria* from the Pacific Coast [24,42], which occasionally mate with each other (S. LePage, Marine Research and Educational Products, Carlsbad, CA, personal communication); and *A. fasciata* and *A. depilans* from the Mediterranean Sea, in which egg-laying and mating aggregations have been repeatedly observed [1], although interspecific mating has not. These results suggest that in aggregations containing multiple animals, attractin might be at least partially responsible for stimulating the formation of copulatory chains and rings that have been observed in the field.

Even Aplva attractin from a genetically distant *Aplysia* species, *A. vaccaria*, is attractive to *A. brasiliana* [39]. The attractiveness of Aplva attractin to *A. brasiliana* suggests that despite only ∼40% sequence conservation in some cases, *Aplysia* attractins share a common 3D structure and receptor-binding site that is probably in the conserved C-terminal helical domain. We hypothesize that the attractins are part of a signaling/chemotaxis mechanism that is common to many marine organisms.

The benefits derived from the aggregation of multiple *Aplysia* species may exceed those derived solely by accessing potential mates. One benefit may be defense from predators. Consistent with this hypothesis, other communal animals that are thought to herd for defense may form aggregates composed of multiple species [7,17,18,33]. Secretion of a pheromone that attracts individuals of a different species may still be useful in attracting a potential mate, if the individual that is attracted subsequently lays eggs and releases an attractin signal that attracts a conspecific. The concentration of attractin released from the egg cordons of multiple individuals should be higher than that from a cordon laid by a single individual. Since Aplca attractin is partially degraded within 30 min of eluting from egg cordons [38] (Fig. 3), a higher concentration of active attractin may be sustained over longer distances, thereby increasing the possibility that additional individuals will be recruited to breeding aggregations.

4.4. Attractin acts in concert with other water-borne pheromones to stimulate attraction

T-maze attraction assays have shown that Aplca attractin acts as part of a bouquet of water-borne odors. This is indicated by the observation that while egg cordons are attractive in the absence of *Aplysia*, attractin is attractive only when *Aplysia* are part of the stimulus [37]; the animal does not need to be a conspecific. The concentration of attractin that is attractive to conspecifics and induces the potential mating behaviors is in the picomolar range normally observed with pheromones, demonstrating that attractin has pheromonal activity [38]. Enticin and temptin, two additional water-borne protein pheromone candidates that are released during egg laying have been characterized; when combined with attractin, the blend of these three albumen gland proteins is

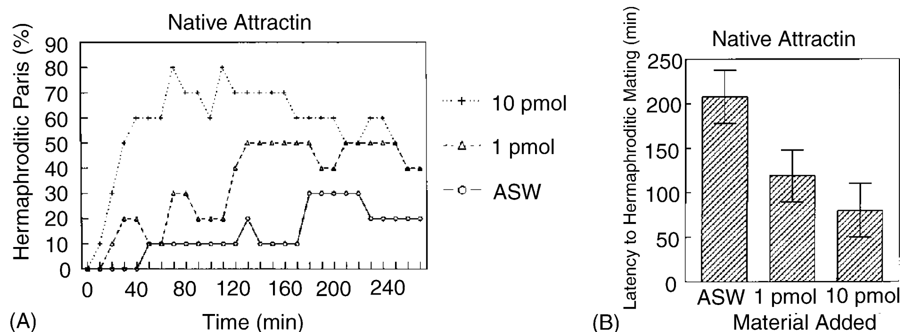

Fig. 6. Aplca attractin induces hermaphroditic mating in *Aplysia brasiliana*. (A) The percentage of animals mating as hermaphrodites was increased when native Aplca attractin was placed in the adjacent seawater. (B) The latency to hermaphroditic mating was reduced by placing either 1 pmol or 10 pmol native Aplca attractin in the seawater.

attractive to *A. brasiliana* [13]. Attractin acts in concert with enticin and temptin to stimulate mate attraction [13]. A movie showing a T-maze attraction assay can be found in the online Supplementary Information section (Movie 2).

5. Conclusions

Attractins form a family of structurally homologous proteins, each of which is sequence-specific for a given opisthobranch species. Nevertheless, attractin is recognized by, and attracts individuals from, other species due to structural similarities among different family members, specifically the IEECKTS domain. Even the distantly related Burle attractin sequence retains the conserved sequence EEC, further supporting attractin mutation studies that demonstrate that the three charged residues in the IEECKTS domain are important for attractin activity. In contrast to the water-borne peptide pheromonal attractants in amphibians, which are species specific [21,22,55,60], the attractins are, to our knowledge, the first peptide/protein pheromone family in invertebrates and vertebrates that are not species specific. The combined structural and behavioral observations suggest a potential mechanism to explain why attractin is not a species-specific pheromone. They open the door to studies of the *Aplysia* olfactory system, an important sensory modality that has not been studied in detail, and provide insights into studies to characterize the attractin receptor. The attraction and mating data demonstrate that attractin may contribute to the establishment and maintenance of breeding aggregations, and to successful reproduction.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.peptides.2004.07.017.

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