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Mini Review

Trypsins: Keystone Enzymes in Estuarine Invertebrate Communities

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Abstract

This review provides insight into the roles of trypsin and its peptide products in signaling and communication in marine animals and in organizing marine communities. Examples include predator attractants, shell cues for hermit crabs, larval settlement cues for gregarious animals like barnacles and oysters, larval release pheromones, curing and signals from biological glues, biofouling management, feeding stimulants and mechanisms of deposit feeding, body odors and molecules that organize breeding aggregations, I argue trypsin should be considered a keystone enzyme because it and its products are central to functioning of marine ecosystems.

INTRODUCTION

Keystone species is an ecological concept that describes a species that is essential to community function and stability [1]. Zimmer and Ferrer [2] expanded the concept to keystone molecules, in their instance toxins that also functioned in communication. Similarly, in marine ecology, trypsin should be considered a keystone enzyme. The major difference between a keystone species or toxin and a keystone enzyme is to eliminate trypsin and all of its roles one might have to destroy all life, as we know it.

If Enzymologists, biochemists and physiologists used ecological concepts like keystone species for enzymes, vertebrate trypsins (EC3.421.4) and other trypsins associated with medicine in general would have the keystone modifier. The rich literature on trypsins is repeat with descriptions on their central roles in metabolism and regulation [3].

Trypsin is everywhere. Trypsins are secreted by all tested phyla of eubacteria, bacteria and eucaryotes [4,5]. As marine chemical ecology matures and the field develops an appreciation for the importance of biopolymers, enzymes and hydrolysis products, trypsins and their products will be appreciated as keystone enzymes and signals. My working hypothesis is over evolutionary time trypsin and its products have become central in the functioning of individuals, groups, communities and ecosystems. This review sets the stage for this concept.

CUES AND SIGNALS IN MARINE COMMUNITIES

Life evolved in the ocean. Chemical senses were among the first of the senses to evolve. Trypsins are evolutionarily ancient enzymes, found in all organisms [6]. It should be expected that chemosensory systems using trypsin and its unique information rich products [7] would evolve in the ocean. Here, I cover what is known about trypsin and its roles in chemosensory functions in multicellular organisms in the ocean [8-10]. It is helpful to put trypsins and their products in the context of chemosensation. Throughout this review I use the term trypsins as general term for any endoproteinase that cleaves after arginine or lysine. Marine Chemical Ecology is a nascent field. It is safe to say that trypsins have not been studied in the details that would be satisfying to enzyme biochemists.

TASTE, SMELL, BODY ODORS, PHAGOSTIMULANTS, KAIROMONES AND PHEROMONES

In the broader context of chemoreception, major sensing systems taste and smell, are similar in marine organisms and in terrestrial organisms. Functionally, in all animals, taste is defined as the verification of food and is based upon common small organic constituents of cells usually at millimolar to micromolar effective concentrations. Though we can only guess for taste in other organisms, in man taste is a reductionist sense in which many different molecules and their specific receptors are reduced to 4 sensations of sweet, sour, salty a bitter and two modifiers, umami and the fatty acid taste senses which amplify positive and negative taste sensations. Disrupting living cells generates taste molecules. Trypsins play major roles in degrading exopolymers and cells.

TASTE AND SMELL

Functionally, smell functions in environmental monitoring. In man, the tens of thousands of organic molecules that function in smell maintain unique identity rather than being reduced to a few perceptual categories. Molecules that function in smell have effective concentrations in micromolar to nanomolar concentrations. Smell molecules can be, released, synthesized

or result from degradation of existing polymers by hydrolytic enzymes including trypsin. Odors that are the essence of feces (skatole), and death (putrescine and cadaverine) are biogenic amines, decarboxylation products of amino acids. Other biogenic amines are common neurotransmitters.

PHAGOSTIMULANTS

Phagostimulants are molecules detected by animals that stimulate feeding. Phagostimulants are routinely taste and smell molecules that are perceived by sensory systems peripheral to what would be called a mouth. In arthropods phagostimulant receptors are located on antennules, dactyls, and other specialized appendages and hairs. Functionally, phagostimulants can be tasted or smelled. All phyla of marine organisms from Cnidaria (anemones corals and jellyfish) to vertebrates (fish) have receptor fields that respond to phagostimulants.

KAIROMONES

Kairomones are chemicals released by one species that provide an adaptive benefit to another species. In marine organisms kairomones are very common and include prey and predator odors, habitat odors, and resource cues. Kairomones are poorly characterized in marine systems but include trypsin generate peptides and polysaccharide hydrolysis products with sub-nanomolar potency.

PHEROMONES

Pheromones are molecules released by one member of a species dectedan respond to by another member of the same species. Both the sender and the receiver benefit. Detection can be conscious or subliminal. The best-chemically characterized marine pheromones are small molecules associated with spawning in marine worms and algae. However, trypsin generated peptide pheromones are common. Pheromones are potent with sub-nanomolar effective concentrations.

AIR VS WATER

The consequences with respect to the diversity of molecules participating in information transmission due to the properties of the major carrier fluids, air in terrestrial environments and water in marine and fresh water environments are dramatic. By-in-large, volatile, small weakly charged molecules are the option in air, while any biologically derived molecule could function to transmit information in water.

Although any molecule could function as a signal molecule in aqueous environments, not all molecules have properties essential to efficient signal transmission. Very large biopolymers are ineffective as signals because an efficient receptor has finite affinity for its signal and bonding interactions determine that affinity. Too many bonds result in to high affinity and poor signal qualities. High biological stability results in signals lasting too long. However, hydrolysis products of biopolymers have features that enable functioning as signals. In marine systems, only trypsin hydrolysis products are well studied.

EXAMPLES OF TRYPSINS AND SIGNALS

Shell cues

Studies of the role of trypsin in generating signal molecules began in the 1970s with investigation of hermit crab attraction to predation events where a large predatory snail was eating a smaller snail. Hermit crabs were attracted to the predation event, not for food, but for the potential of a new shell [11]. The response was very specific with different species of hermit crabs attracted to different species of prey snails [12-14]. Hermit crabs were not attracted to food or mollusks that did not have shells crabs could use. Artificial predation sites generated by mortally wounding a snail gave the same specificity as a natural predation site. However, hermit crabs usually came to natural predation sites within a half an hour while it took hours to attract hermit crabs to an artificial predation site.

Rittschof [14] was curious about the role of the predatory snail and in understanding how the chemical shell cues could be so specific. Using dialysis bags to restrict the size of attractants from flesh Rittschof reported freezing and thawing snail flesh caused rapid attraction of crabs as did treating fresh snail flesh with purified bovine trypsin. Hermit crab responses to fresh snail flesh were slow and responses to fresh flesh with trypsin and added soybean trypsin inhibitor were also slow. All data pointed to small peptides released by the action of trypsin on snail flesh. The puzzle was solved when Rittschof et al. [15], showed that predatory snails lubricate their radula, a tissue tearing structure used in feeding, with digestive enzymes including trypsin and cues are generated at natural predation sites by this mechanism. Interestingly, although snail muscle is the source of peptide attractants, the hexapeptide released during conversion of trypsinogen to trypsin attracts one species of hermit crab [16]. Peptides release by trypsin activity during feeding and snail death and generation of peptides by endogenous trypsin released by freezing and thawing are all likely to occur in nature.

The only recent work in the hermit crab shell cue detection system is identification of a volatile water solubile molecule found in hermit hemolymph which attacts hermit crabs with poor fitting shells to sites where a conspecific died a violent death [17]. This report is fascinating because the active volatile compound was isolated due to its specific binding to an any drotrypsin afinity column. The column was used because the hemolymph molecule was thought to be a trypsin generated peptide. A logical extension from this observation is a pathway for external signals to be used in terrestrial communication.

PREDATORY SNAIL ATTRACTANTS

In the late 1970s predatory snails, oyster drills, were implicated in the decline of commercial oyster fisheries in the US Mid-Atlantic region. Melbourne Carriker, Langley Wood, and Michael Castagnawere funded by US Sea Grant to investigate the attraction of oyster drills to prey. The project supported the training and research efforts of two post docs and graduate students. Key to the ultimate success of the project was a retired volunteer world class peptide chemist, Robert Shepherd. Our goal wasto isolate and purify molecules that attracted oyster drills to prey. Preliminary studies using millions of juvenile oyster drills provided information on the sources of the most potent attractive odors which were from living barnacles [18]. The research enabled studies that indicated the attractant molecules were trypsin generated peptides. Activity of these attractants

is destroyed by exposure to a carboxypeptidase specific to arginine [19]. In seawater the peptides have a very short half-life, presumably because bacteria consume them.

The discovery that juvenile predatory snails were attracted exclusively to the trypsin generated odors of barnacles and the observation that in the field, snails consumed virtually all barnacles before they reproduced prompted a question. Why didn't this mass consumption of smelly barnacles result in selective pressure to select for barnacles that did not produce the odors. The answer was simple. Snail attractant was actually a barnacle settlement pheromone [19]. Barnacles that didn't produce the odors couldn't settle gregariously and facilitate sex and reproduction.

Attraction of snails and induction of metamorphosis in barnacle larvae was estimated to be in nanomolar to picomolar amounts, well below levels of molecules that are usually tasted or smelled and in the range of pheromones. A variety of other peptide mediated signalling systems function at similar levels [8].

GREGARIOUS LARVAL SETTLEMENT

Compared to life in land environments, life in the ocean is backwards. On land plants are stationary and animals move. In the ocean, most plants are small and move with currents and animals like barnacles and oysters are stationary, glued to surfaces where they can use the free energy of water flowing by the surface to filter out and eat small plants. For sessile adults, sex is a primary issue. Animals need to be close enough to each other to participate in any of a variety of ways to breed and reproduce. Many sessile animals settle gregariously maximizing the chance of reproducing.

Gregarious settlement is mediated chemically and for barnacles and oysters, small peptides generated by trypsins are settlement pheromones. Settlement pheromones can originate from curing barnacle glue [20] or from the action of trypsins released by bacteria on proteins in the exposed calcium carbonate skeletons of oysters and barnacles [18], or potentially from the action of trypsins on the cuticular or living surfaces of the animals. Although the actual natural settlement pheromones have specificity, synthetic tripeptide mimics like glycil-glycil-arginine induce settlement in both types of larvae [21,22] Studies with pure synthetic tripeptides confirm potency for larval barnacle and oyster metamorphosis. Sequences of purified peptide attractants were determined in 2013 and are archived at the Duke Proteomics Center and available upon written request to ritt@duke.edu. Finally, tripeptides induce metamorphosis in jellyfish [23].

As our studies progressed over the decades, the widespread occurrence of these signaling pathways became apparent. Anemones [24], Mollusks [9,25,26] and crustaceans [8,9] use these signals, pheromones and cues. It is more than a coincidence that vertebrate leukocyte chemoattractants are peptides generated by trypsins [27].

BIOLOGICAL GLUES

Barnacles and many other marine animals attach themselves to surfaces using glue that cures underwater. Glue curing appears related to blood clotting [20]. Trypsins play a central role in vertebrate blood clotting and barnacle glue curing and in the complement cascade, which is part of pathogen defense. In glue curing, the peptides generated by trypsin clipping during activation of structural proteins and enzymes for glue curing function as barnacle larval settlement pheromones[20] and as kairomones by attracting barnacle predators. Crustacean eggs and mollusk egg capsules are highly crosslinked glues. Trypsins degrade these glues and release pheromones and kairomones that organize snail breeding aggrications [28].

LARVAL RELEASE PHEROMONES

In the early 1980s larval release pheromones were discovered in a small estuarine crab [29,30]. Crabs, Lobsters as well as other crustaceans brood embryos in eggs glued to their abdominal appendages [9]. There is no direct connection between the brooding female and the embryos. Communication is via pheromones that originate in the egg mass. During embryo development the pheromones ensure ventilation to provide oxygento and remove waste products from the developing embryos. At egg hatching the pheromones synchronize larval release [31]. Females vigorously contract their abdomens, which assists in egg hatching. After compression females vigorously fan their abdomen delivering larval crabs to the water column. Adding trypsin to the water bathing an ovigerous crab causes detachment and hatching of the eggs [10,32]. There is evidence both embryos and the brooding female releases trypsin to mediate egg hatching and larval release [10,32,33]. Interestingly, trypsin inhibitors induce larval release behavior [15]. Thus, trypsin inhibitors can inhibit enzyme activity and interact with receptors that usually detect trypsin peptide products.

MUD SNAIL BREEDING AGGREGATIONS

Mud snail breeding aggregations are initiated by molecules associated with oysters and by gastropod mollusk egg capsules. It is likely that trypsin generated peptides that are larval settlement pheromones and that attract predatory snails may also attract snails in the spawning season. In the breeding and spawning season, mud snails are attracted to the odors of barnacle, living bivalves, mainly oysters, conspecific and heterospecific egg capsules, and other sources of biological glues like the tips of parchment worm tubes. Initially, snails mate and glue their egg capsules to living structures unlikely to be buried by soft sediments. At the peak of the breeding and spawning season there are aggregations of tens of thousands of snails and large mats comprised of millions of 500-micron diameter egg capsules. Hermit crabs attracted to odors associated with the egg capsule mats are routinely found trapped because they are cemented into the mats by egg depositing snails. The total chemical communication system is very complex and included larval settlement pheromones/kairomones for oysters and barnacles, shell attractants for hermit crabs and egg laying hormone (ELH) proteins found in egg cordons [35]

BIOFOULING MANAGEMENT

Biology growing on surfaces where it is detrimental is biofouling. We wondered how do you keep yourself clean where you can't scratch. Two likely places to look for chemical fouling

management are brooded eggs which rapidly biofoul and die if they are removed from the brooding female and surfaces of animals with exoskeleton. We looked and discovered a suit of enzymes including trypsin, chymotrypsin and lysozyme are found in both locations [32]. A single treatment of detached eggs with mixtures of hydrolytic enzymes including trypsin is sufficient to enable embryo development and egg hatching.

Harvesting biofouling as food is a common strategy used by deposit feeding animals such as fiddler crabs. Feeding stimulants are generate by the action of trypsin and other hydrolytic enzymes secreted on crab legs and detected by chemorecptors on the tips of the legs [32,36]. Fiddler crabs combine mechanical cleaning with enzymes to harvest between 65 and 90% of the microorganisms attached to sand grains in seconds. Differences in mixtures of enzymes can contribute to sexual dimorphism in feeding activity and differences between species for optimal foraging [37].

INDIVIDUAL BODY ODORS

The flip side of biofouling management using enzymes is generation of body odors. Because proteolytic enzymes attack any correct amino acid sequence they encounter, all proteins of any origin available on the outside of any organism can release peptides and hydrolytic products from biopolymers providing body odors that are species and even individual specific. Body odors are important in organizing marine communities, acting as aggregation pheromones, functioning in species recognition, mechanisms for predator avoidance and prey detection and enabling individual recognition in aggressive interactions. The common but highly specific secretion of trypsin and other hydrolytic enzymes by bacteria and animals provides circumstances and molecular mixtures that can evolve into information streams through natural selection

Trypsins are everywhere in marine environments. They function in feeding, aggregation, resource location, biofouling management, curing of natural glues, pathogen defense, signaling between and within species and organizing of marine communities. It is likely that trypsins and their products are just the first of several key enzymatic pathways that organize and enable marine communities. It is worth considering that it is biochemical pathways, potentialy as much as species that are the procuts of evolution.

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