NOVEL ROLE OF TRYPSIN IN ZEBRAFISH

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It has been shown previously in our laboratory that zebrafish produce trypsin from their gills when they are under stress, and this trypsin is involved in thrombocyte activation via PAR2 during gill bleeding. In this study, I investigated another role of the trypsin that is secreted from zebrafish. This investigation has demonstrated a novel role of trypsin in zebrafish. Not only did this investigation demonstrate the role of trypsin in zebrafish behavior, but also it showed that PAR2 might be the receptor that is involved in trypsin-mediated behavioral response. In addition, we have shown that Gq and ERK inhibitors are able to block the trypsin pathway and prevent the escaping behavior. Finally, the results of this investigation suggest that the cells that respond to trypsin are surface cells, which have an appearance similar to that of neuromast cells.

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INTRODUCTION

It has been known for a long time that animals produce chemicals in response to a variety of cues such as attraction, repelling, and reproductive behavior. These chemicals may influence the physiology and development of others in same species. Bethe, in 1932, was able to distinguish this type of chemical, and he used the term ectohormone [1]. In 1959 Karlson and Lüscher replaced this term with the term pheromones [2]. Since then, a wide variety of fish species have been known to secrete pheromones as a response to social behavior, and the scientists have been trying to identify and understand the functions of each pheromone, and the pheromones have also been termed “Schreckstoff” [3]. For example, hormones have been known to play a crucial role as pheromones especially in reproductive responses [4]. Most recently, a study showed that chondroitin sulfate is abundant in fish skin; its breakdown may be elicited by enzymes released upon injury to the skin of the fish, and chondroitin sulfate may be responsible for the fear response of the fish [5].

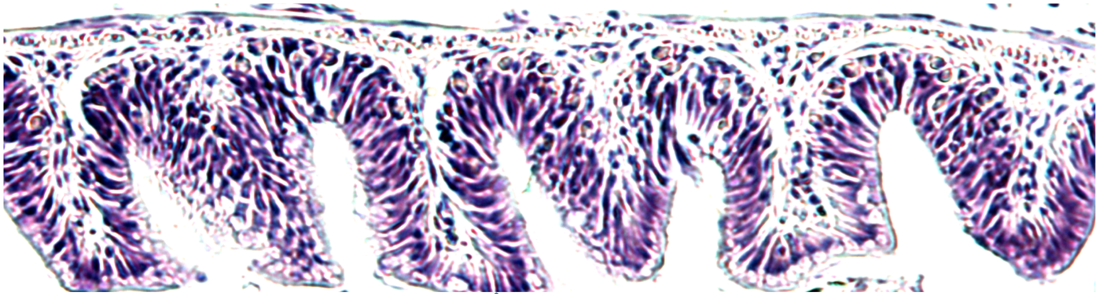
In our laboratory, earlier studies showed that zebrafish produce trypsin into the surrounding water from their gills when they are under stress or injured, and these trypsins protect the gills from bleeding via activation of thrombocytes. Zebrafish trypsin activity also has been shown to be ten times more active than bovine trypsin, and its activity has been found in water ten seconds after exposure to stress or injury. An individual fish upon stress or injury produced on average 0.13 ng of trypsin based on a standard curve generated from purified bovine trypsin. Also, this study demonstrated that trypsin cleaves a protease-activated receptor (PAR) on thrombocytes and initiates a

signaling cascade [6].

Trypsin is a well-known protease that has been shown to be expressed in the pancreatic cells and is involved in protein digestion in the small intestine. It appeared early in evolution in both vertebrates and Invertebrates. In the digestive system, trypsin is generated from trypsinogen (inactive form) in the pancreatic juice, which, following secretion, is activated to trypsin in the small intestine. In the small intestine, the enzyme enteropeptidase, also called enterokinase, is produced from cells of duodenal wall and then cleaves and activates trypsinogen into trypsin. Trypsin is also capable of converting other inactive pancreatic digestive zymogens such as chymotrypsinogen, procarboxy-peptidase, proelastase, and prolipase into their active forms (Figure 1). Trypsin exerts its action as a serine protease that contains a catalytic triad consisting of three amino acids, histidine-57, aspartate-102, and serine-195, in the active site. These three amino acid residues make the active site serine nucleophilic [7]. Interestingly, it has been shown that trypsins are forerunners for several serine proteases such as coagulation proteins [8], [9].

In addition to being expressed in pancreatic cells and being involved in protein digestion, trypsins are also secreted from other extrapancreatic cells such as endothelial cells, epithelial cells, and tumor cells [10], [11]. Fore example, it has been documented that trypsin affects proliferation, invasion, and metastasis of breast cancer cells [12]. Also, a study of the Japanese eel has shown that trypsin is involved in reproductive events in the male eel [13].

Small intestinal epithelial cells



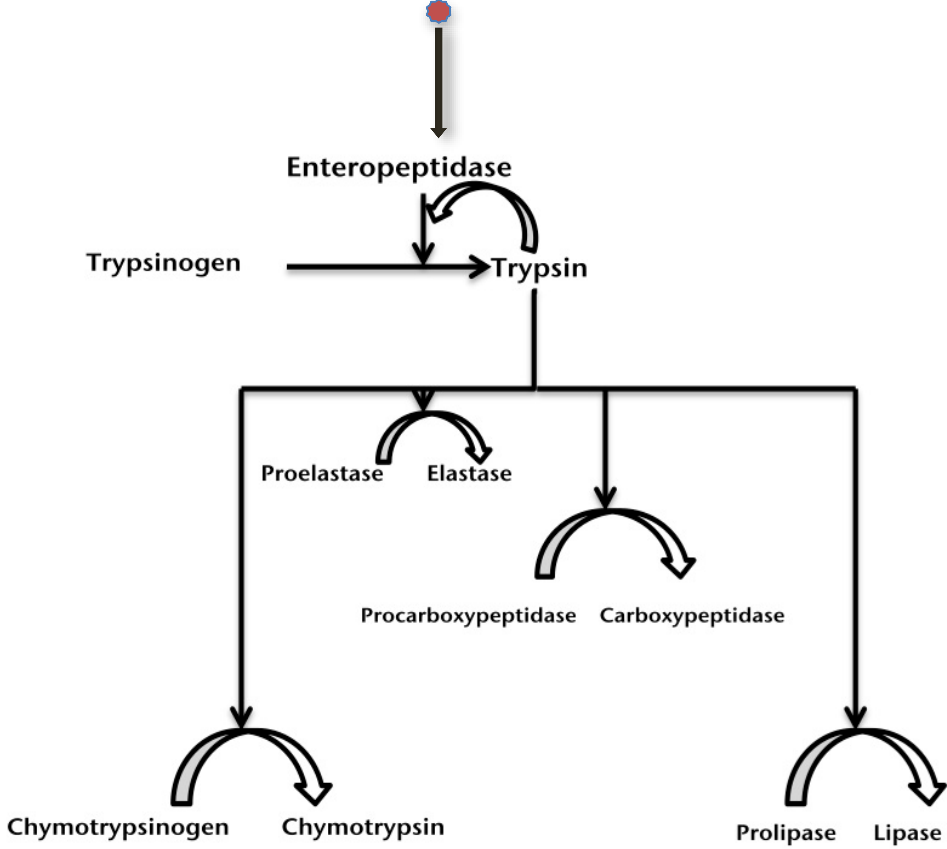


Figure 1. Mechnism of trypsin generation. Trypsinogen is activated into trypsin by the enzyme enterokinase, which is embedded in the intestinal mucosa, and trypsin activates other proteases.

However, little is known about the mechanism of trypsin secretion from other cells. Importantly, trypsin not only cleaves the extracellular proteins in the digestive system but also cleaves the membrane proteins such as PAR2 in a way similar to that in which thrombin digests other PARs. For example, thrombin activates PAR1 and PAR4 on the human platelet surface. Similarly, trypsin initiates a signaling cascade in enterocytes [9].

The PARs are a family of seven trans-membrane G protein-coupled receptors. Their N-terminal domains are outside the cell membrane; their C-terminal is inside the cell and is associated with G-protein. When the protease cleaves the extracellular N-terminal of the receptor, it generates a new N-terminal that acts intramolecularly as a tethered receptor-activating ligand and initiates the signaling cascade [14].

Since trypsin is released into water from gills under stress, we hypothesized that trypsin may also be involved in communicating with other fish via activation of PAR2 on surface epithelial cells. In this thesis, I was able to demonstrate that bovine trypsin induces escape behavior in the fish when they are exposed to this trypsin. I have also used inhibitors that block the PAR2 signaling pathways for trypsin and demonstrated that some of these inhibitors block the escaping behavior. Also, I found that the surface cells, which appear like neuromast cells, are the cells that respond to the trypsin.

PROJECT DESCRIPTION

Zebrafish (*Danio rerio*) have been used widely for a long time as a powerful model system to study vertebrate development. The zebrafish model has led to greater understanding of the mechanisms in both cellular and molecular levels and expanded our understanding of many fundamental biological processes. Recently, zebrafish have also been used to study neuronal behaviors.

The lateral line system is a hydrodynamic sensory system in aquatic vertebrates and is involved in a variety of behaviors in zebrafish. This system includes two types of neuromasts, canal neuromasts and superficial neuromasts. These neuromast cells act as sensory organs that determine the flow of water. In spite of their differences in anatomical locations, their biological functions appear to be similar. Canal neuromast cells are embedded in the sub-epidermal canal, whereas superficial neuromasts lie on the external surface of the fish body. These cells spread over the head, trunk, and tail of the zebrafish. Both superficial neuromast cells and canal neuromast cells include hair cells with kinocilia surrounded by a cupula that is exposed to water flow [15]. However, the cupulae of superficial neuromasts protrude into the surrounding water, whereas cupulae of canal neuromasts are embedded into the canal channel.

Figure 2. A schematic diagram of the lateral line system illustrates the positions of individual neuromast cell clusters shown by closed circles.

Secretion of the trypsin from the gill of the zebrafish into the surrounding water has been recently demonstrated in our laboratory to be involved in the activation of the thrombocytes and then involved in hemostasis [6]. This finding prompted us to think that there may be additional roles for trypsin besides hemostasis. We hypothesized that trypsin may be involved in fish communication. The first question underlying this hypothesis is whether trypsin elicits any behavioral response in fish. If the trypsin were to be involved in fish behavior or communication, other questions follow. Is PAR2 involved? What are the cells that respond to trypsin? To address these questions, I have set up three main aims considering the previous results that we had obtained in our laboratory.

SPECIFIC AIMS

Based on the rationale and description above, the thesis has three specific aims.

Aim 1. The first aim of my project was to expose the zebrafish to the trypsin and observe if there would be any change in the zebrafish behavior. To accomplish this goal I proposed to use zebrafish and expose them to trypsin. Since using the adult zebrafish would be expensive and consume large quantities of our chemicals, I first proposed to perform the experiment on the zebrafish larvae.

Aim 2. The second aim of my thesis was to determine whether blocking PAR2 pathways by using inhibitors would affect zebrafish behavior in response to trypsin. I proposed to use several inhibitors such as Gq protein inhibitor, extracellular signal-regulated kinases (ERK) inhibitor, etc., which are known to inhibit components involved in PAR2 pathways to investigate their capacity to selectively block trypsin action.

Aim 3. The third aim was to identify the type of surface cell that responds to trypsin. To accomplish this goal I proposed to use calcium green (a type of long-wavelength Ca^{2+} indicator molecule that leads to an increase in the intensity of light upon binding Ca^{2+}) release as an indicator to track the type of cell that responds to a trypsin mediated signaling pathway, which triggers release of calcium.

MATERIALS AND METHODS

Zebrafish Husbandry

Zebrafish embryos and larvae were the main components in my research, so taking care of the wild type zebrafish and breeding them were a significant part of my research. The day before breeding, we set up breeding tanks and placed dividers in each individual breeding tank. Then we placed a male on one side of the breeding tank divider and a female on the other side of the same tank. After the room lights turned on early in the morning of the following day, we pulled all dividers out and allowed them to have undisturbed mating time. After 30 min, the eggs were collected from the breeding tanks and kept (as per the typical procedure) in a petri dish containing E3 medium for 4 days without feeding since their G.I. tract had not fully developed. E3 medium is a solution that contains 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1% methylene blue. Beginning 5 days post fertilization, the larvae were transferred from their hatching container and fed with paramecia until they became old enough to eat brine shrimp and fish flakes.

Trypsin Exposure Assay

To test whether there are any significant changes in zebrafish behavior after exposure to trypsin, we designed a novel assay using a multiple channel tray. Using the adult zebrafish would have cost a lot and consumed a lot of our chemicals,

so I preferred to do my experiment on the zebrafish larvae. However, we confirmed the same result in adult zebrafish (data not shown). In this experiment, I used bovine trypsin, which has ten times less activity than the zebrafish trypsin.

Nine dpf zebrafish larvae were maintained under standard conditions. Zebrafish larvae were put in the middle of the multiple channel tray, which has 4.5 ml distilled water so that total amount after adding the larvae would be approximately 5 ml in each channel. Then 20 µL of the 20 nM bovine trypsin was added at the right end of the same channel. In the same tray the larvae were added to middle of another channel containing 4.5 ml distilled water so that again the final volume was about 5 ml, and then 20 µL of the 20 nM bovine serum albumin (BSA) was added to the right end. The behaviors of zebrafish larvae in each channel were observed, and images were taken. For qualification and quantification, the experiment was repeated many times, and statistical tests were performed to show the significant difference between the control and the experimental groups.

Chromogenic Assay

A wild type zebrafish was kept in a plastic cup containing 15 mL distilled water for 10-20 seconds, and then 195 µL of the zebrafish water (ZW) was collected. The same fish was then removed and kept in 1 L of distilled water for 30 min. After 30 min the same fish was put back in another cup of 15 mL distilled water containing 5 µL 20 nM of bovine trypsin after the collection of a 195 µL sample from the cup. This fish was kept for 10-20 seconds, and then again 195 µL of the ZW was collected.

Using these samples, S-2238 cleaving activity was assayed. The assay was performed using 5 μL of substrate in 195 μL of these samples in a final assay volume of 200 μL containing 500 μM S-2238 in 50 mM Tris-HCl pH 7.2 (S-2238 is a thrombin substrate, but trypsin and other serine proteases also cleave this chromogenic substrate, and since it has been used in earlier studies on trypsin from our laboratory, I used this substrate) [6], [16]. Water was used as control. The yellow color due to the cleavage of S-2238 substrate by trypsin was measured at 405 nm in a 96-well kinetic microplater reader.

Trypsin Inhibition Assays Using Different Inhibitors

Gq, ERK, C-src, Phosphatidylinositide 3-kinases (PI3K), protein kinase A (PKA), and protein kinase C (PKC) inhibitors as wells as indomethacin were used. For all the inhibitor experiments, larvae were incubated with appropriate concentrations of inhibitors as described below. Larvae were incubated with the inhibitor for 30 min in final assay volume 500 μL. An incubation time of 30 min was chosen after different incubation times were tried.

All larvae were then transferred to the middle of the channel, and 20 μL of the 20 nM bovine trypsin was added at the right end of the channel. For control we used same number of larvae, which were not incubated with inhibitor, in another channel in the presence of bovine trypsin to compare their behavior. The behavior of the fish was photographed a few seconds to one minute after their exposure to bovine trypsin. We used a final concentration of 250 nM for Gq, ERK, C-src, and PI3K inhibitors.

For PKC inhibitor, we used 36 μM, whereas for PKA we used 5 μM concentration. For indomethacin, a concentration of 50-60 μM was used.

Calcium Green Microinjection

Batches of single cell-stage zebrafish embryos were obtained from the zebrafish wild type line. These embryos were put in a 1% agarose petri dish with grooves or furrows to hold the embryos in place during microinjections, and the microinjection needle was prepared by using vertical pipette puller. This needle was back loaded with 3 μL of 0.1 mg/100 μL calcium green. Then each embryo was microinjected with 5-10 nL of the 0.1 mg/100 μL calcium green under a microinjection microscope. Some uninjected embryos were kept as controls. All embryos were then moved out of the holding dish back into the fresh E3 medium and manipulated by standard methods. Five to nine dpf, each single larva was screened for cells positive for calcium green under a fluorescence microscope to ensure proper delivery of the calcium green compared to controls. The calcium green positive larvae were kept for the next experiments.

Calcium Release Assay

Calcium green (microinjection) larvae were placed, one on each microscopic slide containing a thin film of agarose after anesthesia with Tricaine solution (6 μL of 10 μM Tricaine in 500 μL of water) to ensure immobilization of the larvae. The slide containing a thin film of agarose was prepared by placing 500 μL of 1% agarose on a microscopic slide and then placing another slide on top of the agarose.

Once the agarose had solidified, the top slide was removed gently so that a thin film of agarose was left behind. To the anesthetized larva on the agarose film, 3 μL of the bovine trypsin were gently added. Images were taken before and after bovine trypsin addition, and the intensity of green fluorescence due to calcium green was photographed. The intensities were measured using Image J software.

RESULTS

Escaping Behavior of Zebrafish in the Presence of Trypsin

In previous studies from our laboratory, Kim et al. have shown that trypsin or trypsin-like protease is secreted from a fish's gills when the fish is under stress or injured [6]. They also demonstrated that secreted trypsin is involved in hemostasis. We hypothesized that trypsin may be involved in communicating with other fish via activation of PAR2 on surface epithelial cells. To test this hypothesis and determine if fish respond to the trypsin, I used bovine trypsin. 12-17 wild type larvae were placed in the middle of the channel containing water. Then immediately bovine trypsin was gently added to the water in the channel at the right. Interestingly, approximately 85% of the larvae escaped away from the trypsin side to the opposite side, whereas in presence of the bovine serum albumin as control, in another channel, larvae were moving randomly without any preference to the right or the left side. The movement was photographed after a few seconds to one minute. A representative photograph is shown in Figure 3. The results of six independent experiments are shown in Table 1. We performed similar experiments with adult zebrafish in a bigger tank, and we observed identical behavior (data not shown).

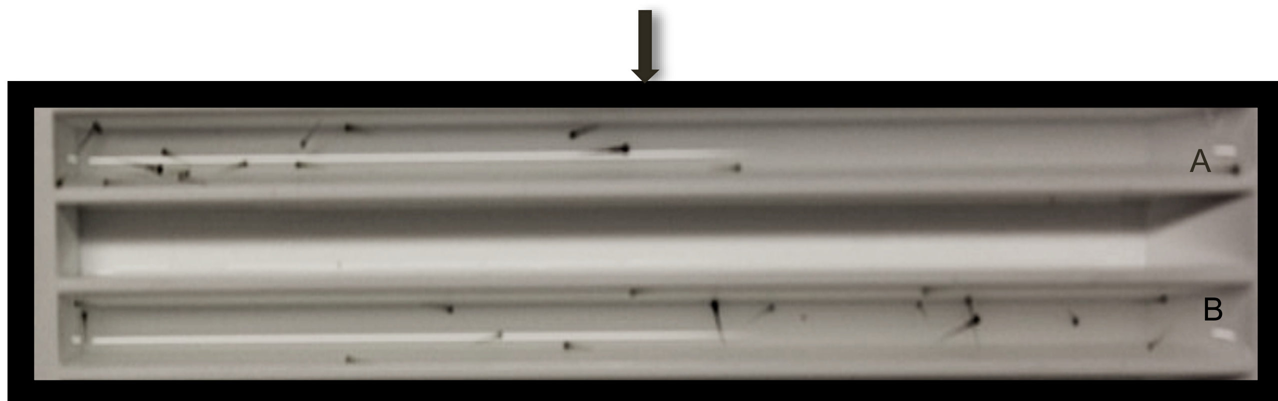


Figure 3. Trypsin exposure assay. Fifteen 9 dpf larvae were placed at the middle of each channel at the point shown by the arrow. 20 µL 20 nM bovine trypsin was added at point A. 20 µL 20 nM of BSA as a control was added at point B.

Table 1. Percentage of larvae that show escaping behavior and go to the left side of the channel in the presence of trypsin (away from trypsin).

Experiment	No. of larvae	Left side	Right side	Percentage %
1	15	13	2	86
2	15	12	3	80
3	17	14	3	82
4	12	10	2	83
5	13	11	2	84
6	16	15	1	93

To ensure that the group escaping was not due to the phenomenon of natural schooling or shoaling behavior, I tested the behavior of individual larva after exposing them to bovine trypsin. In this experiment, bovine trypsin was added on the left side instead of the right side. We obtained similar results as were obtained in our previous experiment where we placed a group of larvae in the middle of the channel. The results are shown in Table 2.

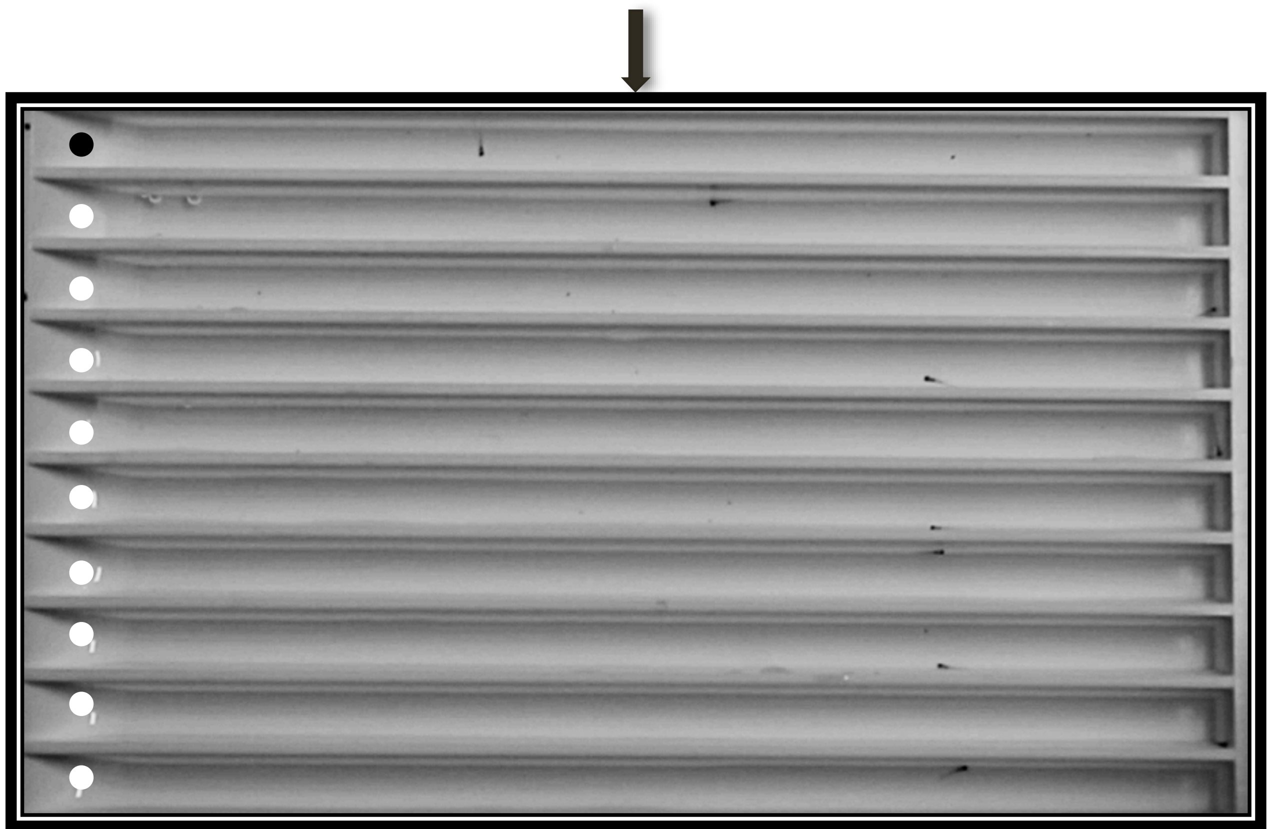


Figure 4. Behavior of individual larva after exposure to trypsin. The arrow shows the place in each channel where the larva was placed. White solid circles denote the places where trypsin was added. Black solid circle denotes the places where BSA was added as a control.

Table 2. Behavior of single larvae in the response to bovine trypsin.

Experiments	Right side	Left side
1	NO	YES
2	NO	YES
3	NO	YES
4	NO	YES
5	NO	YES
6	NO	YES
7	NO	YES
8	NO	YES
9	NO	YES

Induction of Zebrafish Trypsin Release Through the Use of Bovine Trypsin

To determine if the trypsin would induce the fish to produce more trypsin, the following experiment was performed. In this experiment I used serine protease substrate S-2238 (12). A single zebrafish that was placed in water was exposed to bovine trypsin, and the resulting water was assayed for release of new trypsin, which was expected to be above the background levels.

Carefully designed controls for basal trypsin levels secreted by fish as well as bovine trypsin controls, when subtracted from the experimental values, revealed that fish secrete almost five fold greater levels of trypsin than the basal levels.

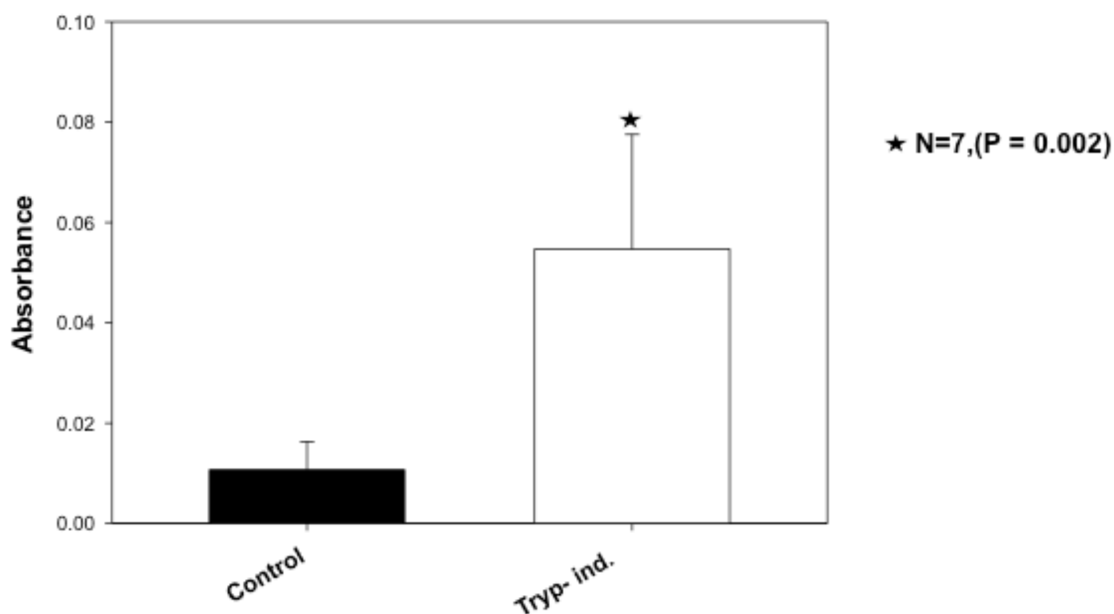


Figure 5. Treatment of zebrafish with bovine trypsin led to induction of trypsin secretion. The absorbance was at 405 nm.

Identification of the Inhibitors that Block the Trypsin Pathway

Since PAR2 has been shown to be preferentially activated by trypsin, and the PAR2 downstream pathway is already known in some mammalian cells, we hypothesized that preincubating the larvae with the PAR2 pathway specific inhibitors should abolish the escaping behavior of fish in response to trypsin.

To test this, I used seven inhibitors, namely Gq, ERK, C-src, PI3k, PKA, and PKC inhibitors as well as indomethacin. Interestingly, only Gq and ERK inhibitors blocked the escaping behavior of fish in the presence of trypsin, and therefore, larvae were moving randomly similar to control larvae, whereas other inhibitors did not alter this escaping behavior.

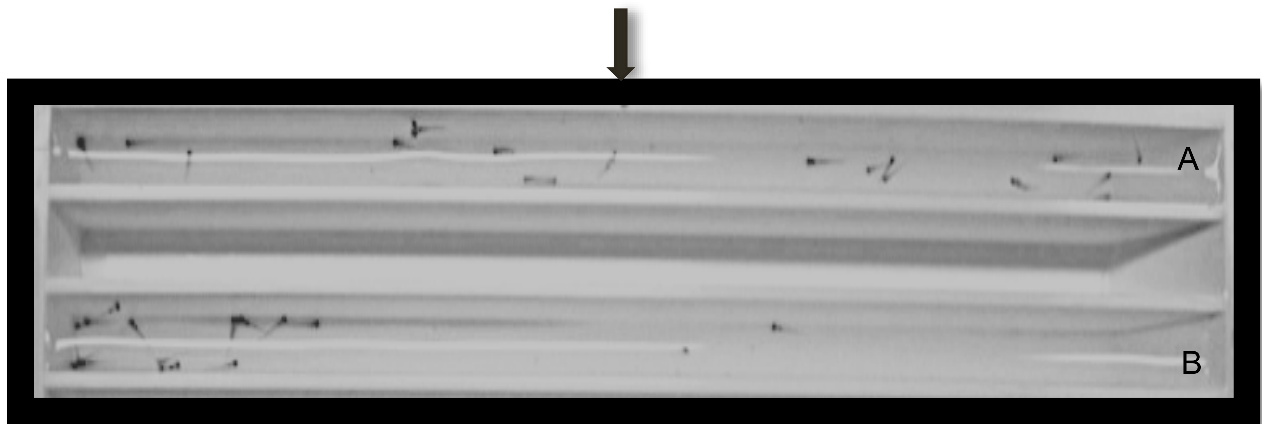


Figure 6. Sixteen 9 dpf larvae were incubated in Gq inhibitor for 30 min and then were placed at the middle of the A channel at the point shown by the arrow. Then 20 µL 20 nM of bovine trypsin was added at point A. In the B channel, only 20 µL 20 nM of bovine trypsin was added at point B as a control.

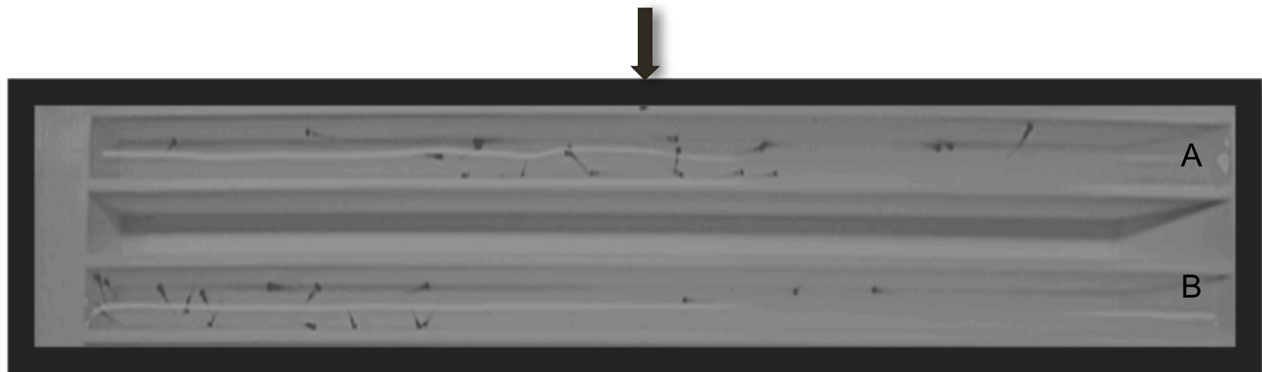


Figure 7. Eighteen 9 dpf larvae were incubated in ERK inhibitor for 30 min and then were placed at the middle of A channel at the point shown by the arrow. Then 20 µL 20 nM of bovine trypsin was added at point A. In the B channel, only 20 µL 20 nM of bovine trypsin was added at point B as a control.

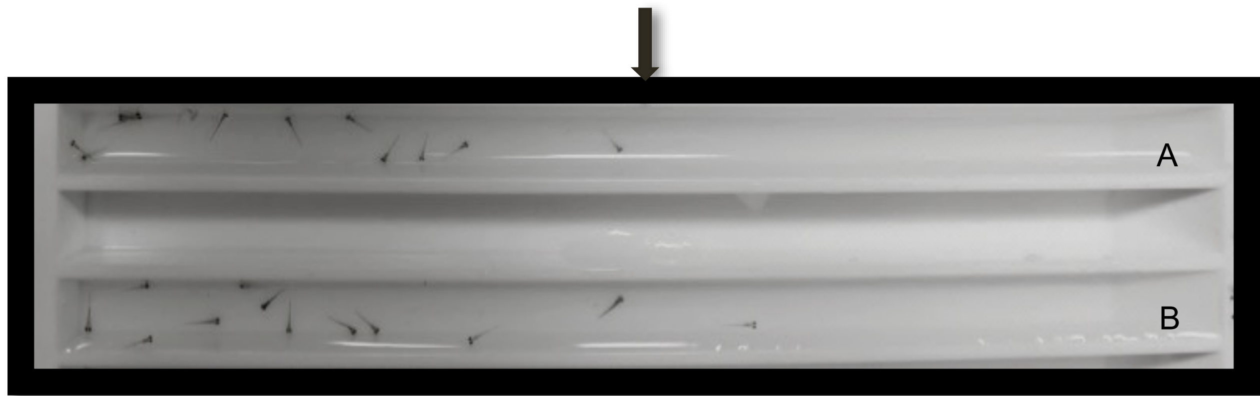


Figure 8. Twelve 9 dpf larvae were incubated in C-src inhibitor for 30 min and then were placed at the middle of the A channel at the point shown by the arrow. Then 20 µL of 20 nM of bovine trypsin was added at point A. In the B channel, only 20 µL 20 nM of bovine trypsin was added at point B as a control.

Table 3. Inhibition of the larvae response to trypsin after incubation of larvae for 30 min in each inhibitor.

Chemical	Inhibition of larvae response
Gq inhibitor	Yes
ERK inhibitor	Yes
C-src inhibitor	No
PI3K inhibitor	No
Indomethacin	No
PKA inhibitor	No
PKA inhibitor	No

Calcium Green Intensity Assay

Since trypsin mediated PAR2 action in some cells is coupled to intracellular Ca2+ release, we hypothesized that observation of calcium release in surface cells may identify the type of cell based on which cell releases calcium upon trypsin treatment. To test this hypothesis we injected single cell-stage embryos with calcium green, and the resulting larvae were selected first for the green fluorescence; then the calcium green positive larvae were treated with trypsin as described in the methods section. We observed increases in the calcium green signal in several areas of the body surface, and all of them were clusters of large cells which had resemblances to surface neuromast cells. The fluorescence intensity of these cells was measured before and after trypsin treatment, and we found that trypsin treatment resulted in significantly greater fluorescence compared to that found in the same cells before treatment.

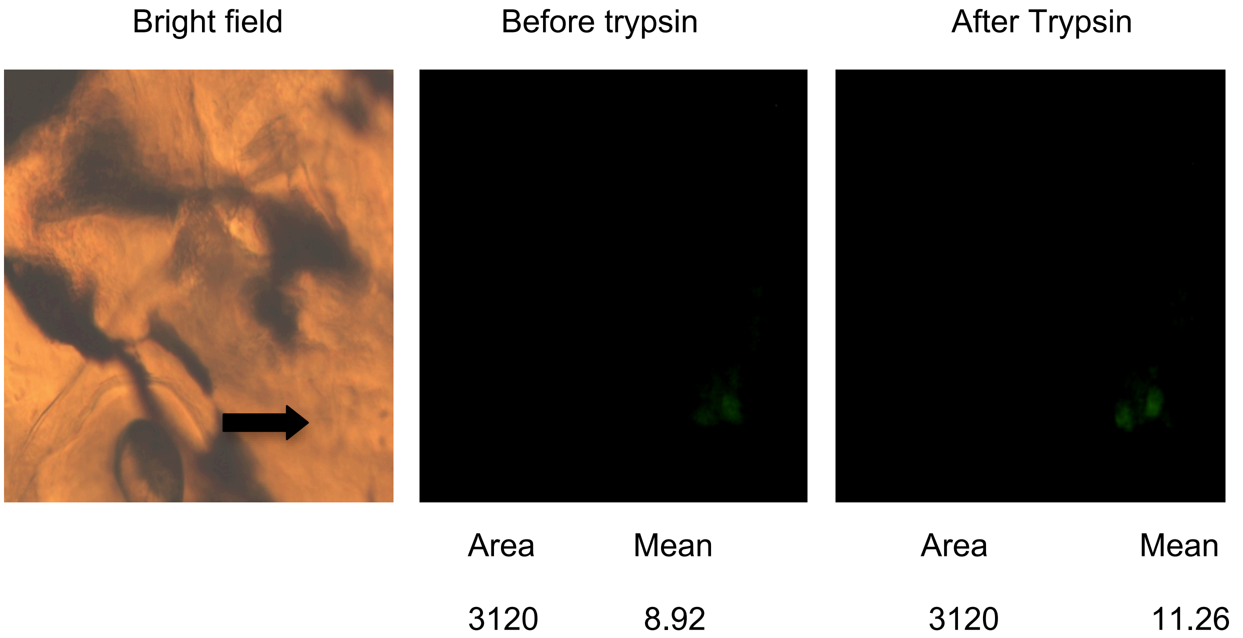


Figure 9. Increase in calcium green intensity in surface cells after exposure to trypsin

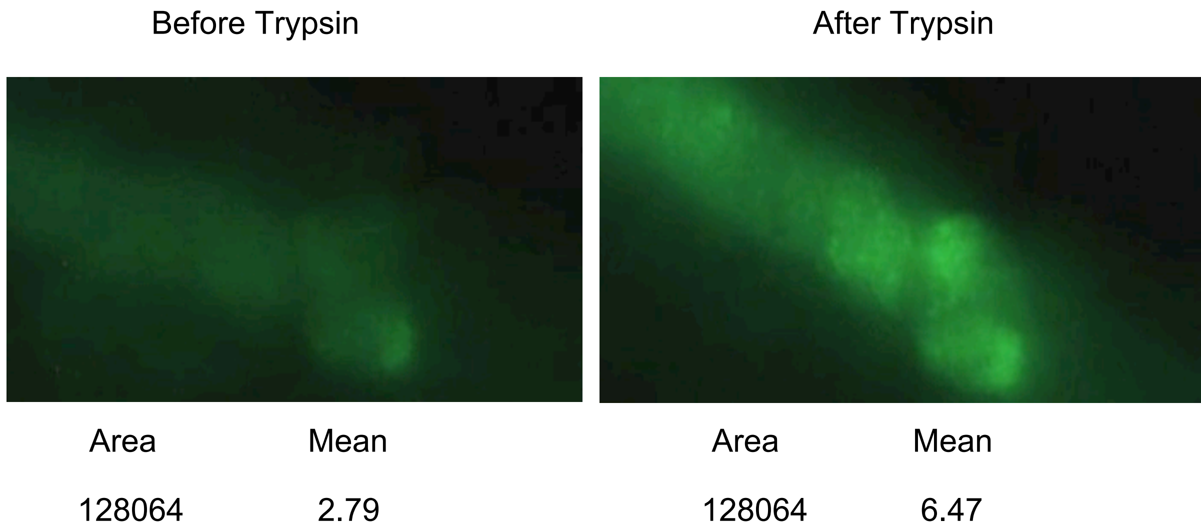


Figure 10. Increase in calcium green intensity after exposure to trypsin.

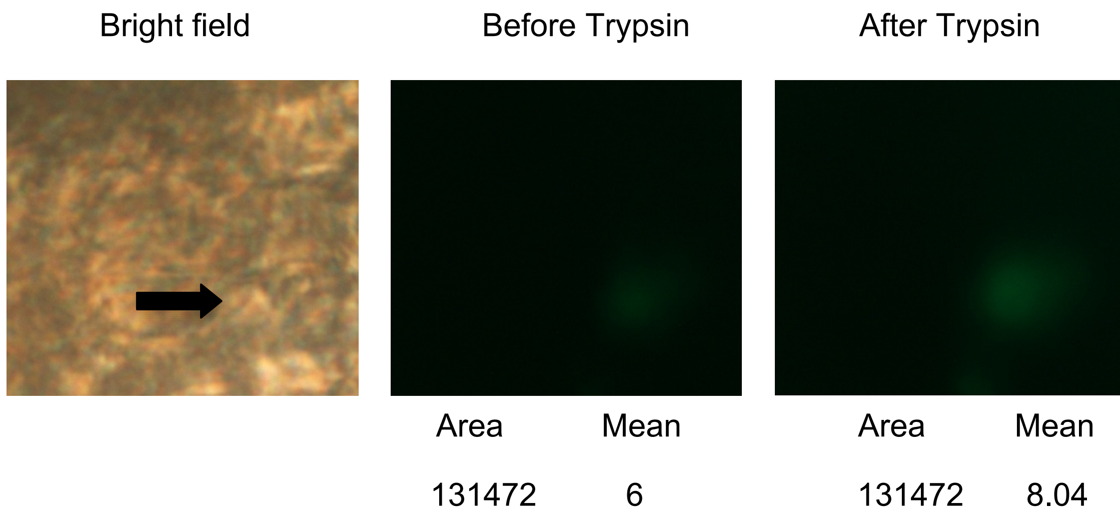


Figure 11. Increase in calcium green intensity after exposure to trypsin.

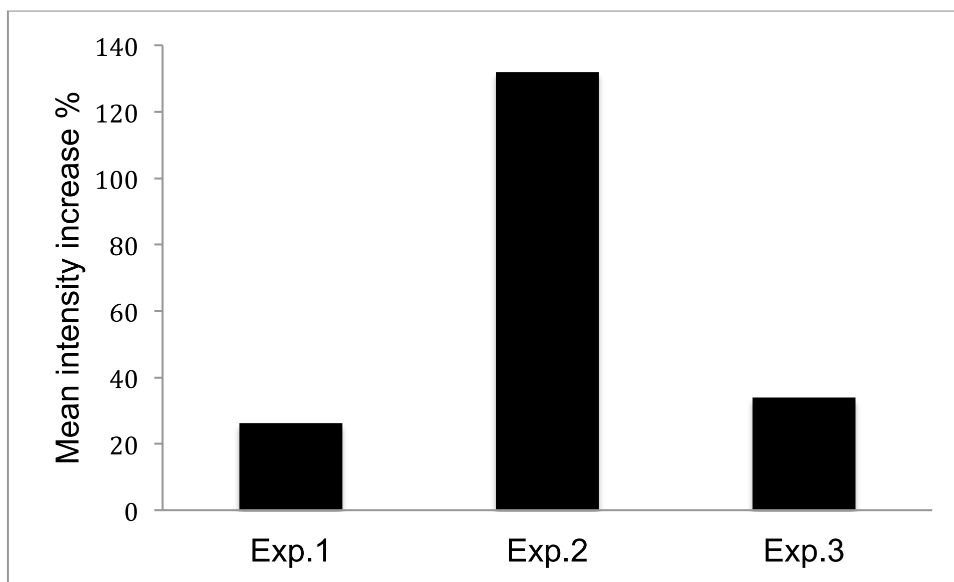


Figure 12. Calcium green mean intensity increase after trypsin exposure.

DISCUSSION

The investigation in this thesis not only demonstrates that secretion of trypsin into surrounding water may regulate fish behavior but also shows that PAR2 may be the receptor that is involved in the trypsin signaling cascade. Interestingly, a recent study showed that fish produce chondroitin sulfate when they get injured, and this initiates a fear response in the neighboring fish [5]. However, this may not explain the communication between the fish when they are under stress. Furthermore, the injury has to be adequate to release chondroitin sulfate, so minor injuries may not release sufficient quantities of chondroitin sulfate. Thus, this type of communication may be a special case pertaining to the release of chondroitin sulfate and may not be generally applicable to responses due to stress. In my thesis I describe a novel mode of communication via an enzyme.

Furthermore, the results of using several cell-permeable inhibitors suggest that Gq and ERK are involved in PAR2 mediated pathway of signaling. In addition to that, they also suggest that cells, which respond to trypsin exposure, are surface cells since the inhibitors may not penetrate deeply into the fish body. Although the calcium green experiment determined calcium green elevation after exposure to trypsin in cells appearing like neuromast cells, we can not conclude that the cells that respond to the trypsin are neuromast cells. Others have used DASPEI dye to stain neuromast cells. However, it remains to be tested whether we can dual label these cells with DASPEI and calcium green. Similarly, other dyes, which will label neuromast cells but have different fluorescence compared to calcium green, must be explored.

The successful finding of such dyes should enable us to dual label the neuromast cells and confirm that calcium green signaling is indeed due to neuromast cells.

In this work, I used a ten times greater concentration of bovine trypsin because it had been shown earlier that fish trypsin is ten times more active than bovine trypsin. This raises an important principle of enzyme-substrate specificity. In fact, this specificity would be useful in species-specific communication. For example, goldfish trypsin may specifically interact with goldfish PAR2, and zebrafish trypsin may interact with zebrafish PAR2 in an effective fashion. Furthermore, if the fish produces more trypsin when in danger or under stress, the greater amount of trypsin that is produced will alert neighboring fish, which in turn will produce more trypsin; thus, the signal will be amplified in a group of fish, and the entire kin will move away. Hence, this amplification may protect the kinship. Interestingly, the function of the basal level of trypsin that is produced is not known and requires further exploration.

FUTURE EXPERIMENTS

Future research should use other dyes which will label neuromast cells but have different fluorescence compared to calcium green or should check whether there is any transgenic line with RFP expressing neuromast cells and conduct trypsin induction experiments. If such a line is not available, it should be generated. Also, more inhibitors should be screened for.

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