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Characterization of Plethodontid
TIMP-like Protein (PTP) and its Role in Tissue
Remodeling During Pheromone Gland Development

By

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and
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Abstract

Plethodon shermani is a species of lungless salamander that has a complex courtship ritual during which the female straddles the male's tail. The male then uses a submandibular mental gland to secrete nonvolatile proteinaceous pheromones to increase females' receptivity to mating. Investigation of the pheromone extract indicated the presence of a protein, termed Plethodontid TIMP-like protein (PTP), with no pheromone-like activity that bears sequence similarity to a group of proteins known as Tissue Inhibitors of Matrix Metalloproteinases (TIMPs). PTP was functionally characterized by measuring its ability to inhibit a range of mouse and human Matrix Metalloproteinases (MMPs). The results of this experiment indicated that PTP is capable of inhibiting at least one human MMP, hMMP-1, a collagenase. Confocal fluorescence microscopy of mental gland cross sections probing for PTP, collagen, and Plethodontid Receptivity Factor (PRF) revealed that PTP is colocalized with pheromones in the mental gland, which helps to explain why PTP is found in the pheromone extract. It is hypothesized that regular PTP secretion during mating acts as a biochemical signal that the mating season is ongoing and that the mental gland should be maintained. Additionally, a recombinant PTP was also expressed in *P. pastoris* for use in further structural characterization.

Introduction

The tissue remodeling process is important to the development of all animals and plays a role in cancer metastasis. Seasonally expressed tissues provide a model to study the tissue remodeling process in a consistent and reproducible way. The red-legged salamander, *Plethodon shermani*, is a terrestrial, lungless salamander indigenous to the mountains of western North Carolina ([Figure 1](#)) that seasonally expresses a submandibular mental gland that produces nonvolatile proteinaceous pheromones used to increase female receptivity to mating. *P. shermani* has been used in previous studies as a model for studying reproductive behavior and pheromone signaling.^[1] The courtship ritual and the pheromones of *P. shermani* have been studied extensively due to their unique nature.^[1-5] During courtship, females will straddle a male's tail in a process known as tail straddling walk. The male will apply pheromones directly to the female's nares using a submandibular mental gland [Figure 2](#). Delivery of the pheromone causes the female to be more receptive to mating with the male due to the effect of two major pheromones: Plethodontid Receptivity Factor (PRF) and Plethodontid Modulating Factor (PMF).^[6] However, a third protein, Plethodontid TIMP-like Protein (PTP), comprises approximately 10% of the extract and does not seem to have pheromonal activity as determined by measuring neuronal activation within the vomeronasal organ (VNO) in females ([Figure 3](#)).^[7]

Using NCBI BLAST, the amino acid sequence of PTP that had been determined earlier was found to be similar to that of Tissue Inhibitors of Metalloproteinases (TIMPs) with an E value of $4 \times e^{-16}$ (hTIMP-1) using NCBI's protein database. Matrix Metalloproteinases (MMPs) catalyze the degradation of structural proteins in the

extracellular matrix such as collagen.^[8] TIMP/MMP binding is responsible for a wide variety of functions including cell proliferation, differentiation, and tissue remodeling.^[9] The expression of TIMPs tightly regulate the location and action of constitutively expressed MMPs in their various roles.^[10] TIMPs are capable of inhibiting a large range of metalloproteinases and are therefore considered broad-spectrum inhibitors. TIMPs are ubiquitous in animals: vertebrates generally maintain four TIMP gene copies compared to a single TIMP in invertebrates. In humans, all four TIMPs inhibit the 23 human Matrix MetalloProteinases (hMMPs) to some level with the exception of hTIMP-1, which has low affinity for the membrane-type hMMPs: hMMP-14, hMMP-16, and hMMP-24 as well as for hMMP-19.^[10, 11] Additionally, the TIMPs are also able to inhibit other non-MMP metalloproteinases such as **A Disintegrin And Metalloproteinases (ADAMs)**, **A Disintegrin And Metalloproteinases with ThromboSpondin Motifs (ADAMTSs)** and other matrix metalloproteinases.^[12] ADAMs and ADAMTS have been implicated in control of membrane fusion, cytokine and growth factor shedding, and cell migration.^[13]

Out of the four vertebrate TIMPs, PTP was found to be most similar to TIMP-1 by comparing the primary sequence of PTP to that of the four vertebrate TIMPs ([Figure 4](#)). However, comparison of the sequences from vertebrate TIMPs, invertebrate TIMPs, and PTP revealed that PTP is more distantly related to vertebrate TIMPs than vertebrate TIMPs are to one another ([Figure 5](#)). Because of its similarity to the TIMPs, PTP was hypothesized to have TIMP-like activity and possibly facilitate mental gland development. After primary sequence comparison and functional characterization as an MMP inhibitor, it was determined that PTP has TIMP-like activity and based on the data, it likely results from an ancient gene duplication that predates the other TIMP divergence.

The location of PTP expression within the mental gland was unknown. It was therefore difficult to hypothesize the exact mechanism for PTP action in mental gland tissue remodeling processes. Understanding where PTP is located within the gland and its relative expression over time gives a more complete picture of mental gland development.

As part of the characterization, future studies will investigate the solution structure of PTP using ^{15}N NMR experiments. Since isotopically labeled PTP is necessary for those experiments, a recombinant PTP must be produced. The 12 cysteine residues involved in disulfide bond formation in PTP complicate recombinant protein expression. Typical *E. coli* protein expression strains have reducing cytoplasm that is not conducive to disulfide bond formation and lack an endoplasmic reticulum, which has been shown to be the site of disulfide bond formation in eukaryotes.^[14] Numerous bacterial strains have been developed to address this problem. For example, New England Biolab's SHuffle T7 *E. coli*, which has been modified to express disulfide bond isomerase DsbC and has an oxidizing cytoplasm to allow for disulfide bond formation.

Several attempts using the NEB SHuffle T7 strain were executed under a variety of temperature, media, and IPTG concentrations. Small quantities of rPTP were expressed as a fusion protein with Maltose Binding Protein, which has been shown to have chaperone-like activity.^[15] However, the amount of protein produced was insufficient for further experimentation. In order to express correctly folded rPTP in sufficient quantities, *Pichia pastoris* was utilized as an alternative protein expression vector.

P. pastoris is a species of methylotrophic yeast that was identified as a viable protein expression vector in 1990.^[16] The chemical environment is critical in correct protein folding and disulfide bond formation. Both *P. pastoris* and *P. shermani* are eukaryotic. In eukaryotes, disulfide bond formation takes place primarily in the endoplasmic reticulum. Therefore, expression in *P. pastoris* is more similar to the native protein expression in *P. shermani* than expression in a prokaryotic vector, like *E. coli*, would be. *P. pastoris* has also shown improvements in protein processing, protein folding, and posttranslational modification over *E. coli* and *Saccharomyces cerevisiae*.^[17] *P. pastoris* is also able to secrete recombinant products into the extracellular environment when a signaling sequence is expressed on the recombinant protein. The signaling peptide is not bound to the final product as it is cleaved during secretion.

Figure 1. Distribution map of *Plethodon shermani*.

Figure 2. Prominent submandibular mental gland of a male *P. shermani* during the mating season.

Figure 3. VNO tissue sections treated with a mixture of agmatine and either PBS, as a control, or the male mental gland extract. Upon neuronal activation, agmatine is taken up into the neurons within the VNO. The tissue is then thoroughly rinsed, fixed, and stained for agmatine using an anti-agmatine antibody. The black arrow indicates an activated neuron that is agmatine-immunoreactive. The number of activated neurons can be counted and used as a quantification of the reactivity of the VNO neurons with a substrate. The scale bars are 10 μ m.^[18]

PTP	1	CTCPRPHLQSAFCTSDYVLMVIFQGNPSEDDG-WIRHGVEVHEANKGGT---EALS LDA	56
TIMP-1	24	CTC PH Q+AFC SD V+ F G P + + R+ +++ + KG +A +	83
	57	VYSQTLFG-CGYRHHSQDYTSVYVITAKKENGRAVVDGCSFIKPLYDLIAGQIFGIRGNY	115
	84	VY+ + CGY H S + + ++I K ++G + CSF+ P L Q G Y	143
	116	KKGC-DCTIVPCYASLCDGSQSSNQCFW-DQY--GTSTTSQEVQEYCAKNGEGNCAW	168
	144	GC +CT+ PC + C QS C W DQ G+ Q C G C W	199
		TVGCEECTVFPCLSI PCK-LQSGTHCLWTDQLLQSGSEKGFQSRHLACLPREPGLCTW	

Figure 4. Primary amino acid sequences of TIMP-1 starting at amino acid 24 and PTP starting at its N-terminus. The amino acids written between the two lines represent exact matches between the two sequences while the plus signs represent synonymous substitutions. The proteins share 48% positive residue matches that are either synonymous or identical indicating a structural similarity between TIMP-1 and PTP. The E score is 4e-16.

Figure 5. Gene tree comparing PTP to both invertebrate and vertebrate TIMP orthologs. These data suggest that PTP may represent an ancient gene duplication that predates most vertebrate lineages.

Figure 6. Fluorescence Confocal Microscopy

- (a) PTP expression and localization for mental glands at different stages of development (6/13 and 8/13) using α -PTP (green), α -collagen (red), and DAPI nuclear stain (blue)
- (b) PRF and PTP expression and localization for mental glands by immunohistochemistry using α -PTP/biotin (green), α -PRF/Atto-633 (red) and DAPI nuclear stain (blue).

Specific Aims:

Aim 1: Functional Characterization

As the mental gland has a tightly regulated seasonal development, it has been proposed that PTP could act as a TIMP and inhibit secreted MMPs, which would otherwise degrade the gland. Since PTP is secreted during courtship, it could serve as an indicator that the pheromone has been delivered. Once the courtship season ends, absence of PTP could indicate that the mental gland is no longer needed. Consequently, regular secretion of PTP during tail straddling walk may serve as a unique chemical signal that the courtship season is ongoing. It has been observed qualitatively that isolated males undergo recession of their mental glands. It is likely that expression of PTP is controlled by external signaling pathway such as testosterone levels or RNA-binding proteins as both have been shown to influence mental gland development.^[19, 20]

To better characterize the function of PTP, its activity as an MMP inhibitor was quantitatively assessed. This determination of PTP's ability as an MMP inhibitor was made by measuring the rate of cleavage of the fluorogenic substrate OMNIMMP[®] RED fluorogenic substrate by a variety of human and mouse MMPs with and without the presence of PTP. Upon cleavage, the substrate fluoresces; the amount of fluorescence can be used as a measure of the rate of cleavage. Therefore, information about the enzyme kinetics with and without the presence of PTP can be gathered.^[21] It is important to note that MMPs from *P. shermani* are not available for use in this assay, but the conserved nature among MMPs^[22] and the broad range of TIMP inhibition^[23] served as indicators that inhibition may be observed between a TIMP from *P. shermani* and human MMPs.

Indeed, inhibition was observed when using PTP in conjunction with hMMP-1 indicating that PTP does possess TIMP-like activity.

Aim II: Characterization of the Expression and Localization of PTP

In order to observe the expression and localization of PTP at different stages of mental gland development, immunofluorescence confocal microscopy were employed to visualize PTP (**P**lethodon **T**IMP-like **P**rotein), PRF (**P**lethodon **R**eceptivity **F**actor) —a pheromone, and Collagen II—a substrate for MMP-1.^[24] Antibodies against PTP and collagen were used to detect each protein. DAPI was used to visualize the nuclei. The micrographs were overlaid to visualize co-localization during different points in gland development. The images revealed that PTP is localized with PRF in the mental gland. Collagen indicated the that the extracellular matrix was extensively remodeled during gland development.

Aim III: Expression of rPTP

A structural analysis of PTP will provide further insight into the function of PTP and can be utilized in future studies to examine its ability to bind to various substrates such as MMPs. NMR can provide valuable structural data. In order to obtain NMR data, an isotopically labeled recombinant protein needed to first be obtained. This was first attempted by cloning the PTP gene for overexpression in *E. coli* to allow expression of a recombinant PTP (rPTP). rPTP was expressed as a fusion protein with Maltose Binding Protein, which has previously been demonstrated to increase properly folded recombinant TIMPs in an *E. coli* expression system.^[25] After numerous attempts to optimize the

expression of rPTP in an *E.coli* system, it was concluded that the yield of correctly folded rPTP would be impractically low for pursuing NMR studies. In order to obtain larger quantities of correctly-folded rPTP, its gene was cloned for overexpression in *P. pastoris*. Utilizing this method was promising as *P. pastoris*, a species of yeast, is eukaryotic and thus possesses an endoplasmic reticulum, which has the oxidizing environment in its lumen that is often necessary for the correct folding of other disulfide bonded proteins that are naturally found in eukaryotic cells. This strategy led to the expression of many proteins that could not be expressed in *E. coli* ^[26]. The expression conditions were optimized and ¹⁵N labeled rPTP was expressed using ¹⁵N ammonium sulfate as the nitrogen source in the media. The resulting rPTP was concentrated, and purified via ultracentrifugation and size exclusion chromatography on a Sephadex G75 column.

Further purification was not required since the recombinant protein was secreted into the media which contains relatively few other proteins ^[27]. The structures of PTP and rPTP were compared via reverse phase chromatography and were found to have the same retention times. Reverse phase chromatography is highly sensitive. Even small changes in the conformation of the protein would change its hydrophobicity enough to result in a different retention time. Since the retention times were effectively the same in both cases, rPTP is believed to be structurally believed to have a similar structure to native PTP. Further analysis will be conducted by determining the disulfide bonding pattern. It should also be noted that rPTP was designed to have identical primary structure as PTP without any additional amino acids or modifications that are not found in native PTP.

Materials and methods:

Animal Collection and Gland Removal: *P. shermani* were collected in June and August 2013 from Wayah Bald in western North Carolina. The submandibular mental glands were removed from *P. shermani* during survival surgery under anesthesia. Pheromone was extracted from the gland by incubating with 0.8 mM acetylcholine chloride in Amphibian Ringer's solution. The extract was then incubated for 1 hour followed by centrifugation at 10,000 x *g* for 10 min, the supernatant was removed and re-centrifuged prior to storage at -80°C.

MMP Inhibition Assay: The MMP inhibition activity of PTP was measured using an MMP substrate, OmniMMPTM Red fluorogenic substrate peptide, which fluoresces when it is cleaved. The rate of cleavage is measured by the level of fluorescence. The assay was performed with a negative control (no PTP) a positive control (the chemical inhibitor NNGH) and 5 concentrations of PTP ranging from 20pM to 200nM. The lower concentrations were chosen based off of endogenous levels of TIMPs and wide range was used to account of a decreased association affinity between *P. shermani* PTP and human MMPs. MMP- 1,2,7,8,9, and 13 were tested. The assay was performed in 50mM HEPES, 10mM CaCl₂, 0.05% Brij-35, at pH 7.5. The fluorescence of the reaction was measured every minute for 2 hours, and the rate was quantified by linear regression. The assay was repeated for MMP-1 using a broader gradient of 10 different PTP concentrations from 7.8 nM to 4 μM.

Fluorescent Confocal Microscopy: Mental glands from June and August 2013 were fixed using 4% paraformaldehyde and 8 μ m sections isolated by microtomy. Four slides from each time point were used such that the two antibody treatments were tested in duplicate. One set of slides was treated with DAPI, anti-Collagen, and anti-PTP. The other set was treated with DAPI, anti-PRF, and anti-PTP. The slides were photographed using a confocal microscope.

Synthesis and Refolding of rPTP: Expression of rPTP as a fusion protein with Maltose Binding Protein was performed using an *E. coli* expression system. The construct included a short linker (SGAAAAGGSDP) between the two proteins and a hexahistidine tag sequence on the N-terminus of the Maltose Binding Protein. The PTP coding regions were PCR amplified from mental gland cDNA and confirmed by Sanger sequencing. The amplified sequence was ligated into the pET45b-based vector using T4 DNA Ligase (New England Biolabs). The plasmid was then transformed into T7 Express lysY/Iq chemically competent *E. coli*. Clones were initially cultured in 100mL of LB media with ampicillin (LB/Amp) at 28°C overnight. The culture was then diluted up to an OD600 of 0.05 in 1L of LB/Amp. This culture was grown to a final OD600 of ~0.6 at 28°C at which point IPTG was added to a final concentration of 0.1 mM.

After a 24 hour induction period the cultures were centrifuged at 2,000 x g for 30 minutes at 4°C. The cultures were then resuspended into 2.2 mL of lysis buffer (50 mM NaCl, 50 mM Tris pH 8.0, 2mM EDTA, 0.1% Triton X-100). The lysates were then thoroughly sonicated. 240 μ L of 10mg/mL lysozyme solution was added to each lysate. Sonication was used to disrupt the cell membranes and fragment the bacterial DNA.

Lysozyme was used to enzymatically break down the bacterial cell walls. The lysates were then incubated at 25°C for one hour. The lysates were then frozen at -80°C. The lysates were then thawed and centrifuged at 10,000 x g for 10 minutes. The supernatant was then decanted from the pellet.

The protein was purified on a nickel-chelation column, but disulfide scrambling was observed so the protein was fully reduced and denatured in 4M urea, 0.1% Triton X, 20 mM β ME, 150 mM NaCl, and 50 mM Tris pH 8.0. Refolding was attempted via rapid dilution and dialysis. The refolding attempts produced insufficient properly folded rPTP for further experimentation. Inclusion bodies were not found to include significant quantities of rPTP. Nickel column fractions were also tested for rPTP, but no significant rPTP was present.

Strain selection

Expression optimization included screening two different *P. pastoris* strains: X-33 and KM71H. The two strains differ by their methanol utilization rate. X-33 utilizes methanol at a faster rate than KM71H. KM71H has been designated Mut^S for Methanol utilization slow and X-33 has been designated Mut⁺ for fast methanol utilization. Expression levels of each strain were measured by lysing the culture after one day of induction in 1% methanol. The protein abundance was determined by measuring the absorbance at 280nm. The relative amounts of rPTP were also measured by SDS-PAGE and Coomassie staining. The KM71H strain was determined to give the highest rPTP yield and was used for all subsequent expressions.

Preparing competent *P. pastoris*

X-33 and KM71H *P. pastoris* were streaked on YPD agar plates and incubated at 30°C for two days. A single colony was selected from the YPD plate and used to inoculate a 10 mL YPD culture. The culture was then grown at 30°C to an OD600 of 0.6 to 1.0. The culture was then pelleted at 2,000 x g at 4°C for 5 minutes. The pellet was resuspended in 10mL of Solution I (sorbitol, ethylene glycol, DMSO) provided with the Invitrogen EasySelect™ *P. pastoris* and then pelleted again at 2,000 x g at 4°C for 5 minutes. The pellet was then resuspended into 1mL of Solution I. 100µl of the suspension was aliquoted into sterile 0.6mL tubes. The tubes were wrapped in paper towels and frozen at -80°C until needed for transformation.

Preparing rPTP for transformation in *P. pastoris*:

The yeast shuttle vector pPICZα C, Zeocin, and *P. pastoris* strains X-33 and KM71H were purchased from Invitrogen (Carlsbad, CA) as part of the *P. pastoris* EasySelect™ Expression Kit. pPICZα C and the rPTP gene were digested with the restriction enzymes XbaI and XhoI from New England Biolabs (NEB) for two hours at 37°C. The newly digested vector and insert were purified using a QIAquick PCR Purification Kit. NEB's T4 ligase was used in the ligation of pPICZα C and rPTP at room temperature for two hours at 25°C.

The resulting plasmid was transformed into XL1-Blue competent *E. coli* cells by first incubating the DNA on ice with the *E. coli* for 30 minutes. The cells were then heat pulsed for 30 seconds at 42°C. The cells were incubated in SOC media at 37°C for one hour and then plated onto LB agar with Zeocin added. After approximately 12 hours of

incubation at 37°C, a sample was taken from each colony. The rPTP gene was PCR amplified from the plasmid and the resulting PCR products were Sangar sequenced. A clone with the correct sequence was selected and PCR was used to amplify and linearize the pPICZα C with rPTP insert. Because the pPICZα C and insert were integrated into the *P. pichia* genome through homologous recombination, the plasmid must be linearized before transformation. The plasmid was purified using a Qiagen PCR cleanup kit so that Taq polymerase and primers were removed prior to transformation.

Transformation of rPTP in *P. pastoris*:

50μL of competent X33 and KM71H cells were thawed on ice. 3 μg of plasmid was added to each tube of competent cells. 1mL of Solution II was added to each tube of cells and vortexed. The transformation reaction was incubated at 30°C for one hour and vortexed every 15 minutes. The cells were then heat shocked at 42°C for 10 minutes. The cells were then split into two 1.6mL tubes and 1mL of YPD was added to each tube. After incubation at 30°C for one hour, the cells were pelleted at 3,000 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 500uL of Solution III, a salt solution provided by Invitrogen. The cells were pelleted at 3,000 x g for 5 minutes and resuspended in 100uL of Solution III. These cells were then plated on LB Agar with Zeocin.

Expression of rPTP in *P. pastoris*:

Clones were selected from agar plates and restreaked onto new plates. Each clone was then used to inoculate 25mL cultures of buffered complex medium containing glycerol (BMGY, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol). The cultures were incubated at 25°C for 18 hours. The cells were pelleted at 2,000 x g for 10 minutes at 4°C. Expression was then induced by resuspending the cells in buffered complex medium containing methanol (BMM, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1%, 0.5% methanol). After 48 hours of induction the cells were pelleted at 2,000 x g for 10 minutes. The supernatant was then removed and concentrated by centrifugal ultrafiltration using 15mL Amicon filter units from Millipore with a molecular weight cut off of 10 kDa. A sample from each clone's ultrafiltrate was examined by SDS-PAGE and coomassie staining. One of the KM71H clones was shown to have the highest yield and was used for all subsequent expressions.

To acquire sufficient rPTP for ^{15}N NMR experiments, four liters of *P. pastoris* culture were grown. 500mL of BMGY media were inoculated the selected KM71H clone and incubated for 18 hours at 30°C. The cells were then pelleted at 3,000 x g for 5 minutes. The cells were resuspended in BMM with 1.5g ^{15}N ammonium sulfate per liter to a final OD600 of 0.6 in order to induce expression. The cells were cultured at 30°C for 4 days. The cells were pelleted at 3,000 x g for 5 minutes. The supernatant was concentrated into approximately 30mL by centrifugal ultrafiltration using YM10 ultrafilters from Millipore. The concentrated was purified by size exclusion

chromatography using Sephadex G-75 gel filtration medium resulting in a final ^{15}N -labeled rPTP yield of approximately 6 mg.

Results and Discussion:

Functional characterization of PTP

The primary sequence similarity between PTP and the other TIMPs suggests that PTP is likely the result of an ancient gene duplication. The similarity between the proteins presented a possible function for PTP as an inhibitor of metalloproteinases. In order to assess this, a group of human MMPs and a mouse MMP were selected. The MMPs were chosen to be representative of the categories of proteinases that MMPs fall into such as collagenases, gelatinases, and matrilysins. Using a wide range of MMPs from multiple species along with the broad range of inhibitory activity seen in TIMPs presented an opportunity for inhibitory activity of PTP, if present, to be demonstrated.

([Figure 7](#)) PTP inhibition of MMP-1 was observed both initially and again over a range of PTP concentrations ([Figure 8](#)). Starting multiple trials of the MMP inhibition assay at once proved to be challenging and is likely responsible for some of the difficulties in assessing statistical significance of inhibition with some of the other MMPs. The effect of PTP binding to hMMP-2 presents an unexpected picture with regards to hypothesized TIMP-like activity. TIMPs have not been shown to activate any MMPs. However, TIMPs have been shown to bind to a wide range of MMPs and even pro-hMMP-2 to prevent its conversion to the active hMMP-2. The exact mechanism for MMP-2 activation is uncertain. However, it is an indication of PTP and MMP interaction, which still provides for PTP having a TIMP-like role. Other regulatory proteins have been shown to have inhibitory or activating role depending on the context so the kinetic data raises a dual regulatory role as a possibility of PTP function.^[28]

Localization and temporal expression within the mental gland

In order to determine the localization and expression levels of PTP qualitatively, immunohistochemistry and confocal microscopy were employed. Immunohistochemistry involves the detection of antigens using antibodies. When used in the context of microscopy, antibodies can be labeled with a fluorescent reporter and used directly on tissue sections to reveal antigen localization.

Antibodies were used to label PTP, PRF, and collagen within the mental gland. α PTP and α PRF antibodies had been previously isolated as polyclonal antibodies from rabbit antisera whereas, α collagen is commercially available. In order to clearly visualize each antigen separately, the reporters with which each antibody is labeled were chosen for emission at different wavelengths. The presence of each antigen was then examined using a filter that only allows the wavelength of light to pass through that corresponds to a specific reporter. Confocal microscopy can be used in conjunction with fluorescently-labeled tissue sections to generate images ([Figure 6](#)). Confocal microscopy provides advantages over conventional wide-field fluorescence microscopy in that optical resolution and contrast are improved in confocal microscopy.

Characterization of the Expression and Localization of PTP

The expression and localization of PTP within the mental gland was examined using confocal fluorescence microscopy. The micrographs generated can be seen in [Figure 6](#). Examining the mental gland in June while it is early within its development reveals that in addition to a reduction in the lumen size of the mental gland, PTP is not expressed. As previously stated MMPs play a critical role in the tissue remodeling

process. As the gland development is stimulated through an increase in testosterone levels, MMP activity is likely the mechanism through which the extracellular matrix is broken down to allow for the expansion of the mental gland. In order to maintain sufficient MMP activity for the mental gland to be expanded, PTP expression must be suppressed early in development. As the gland begins to mature, PTP expression is stimulated and its presence can be observed in the sample from August when the mental gland is mature.

PTP is present at a relatively high concentration in the *P. shermani* pheromone extract along with the other *P. shermani* pheromones such as PRF. In order to better understand how PTP is localized in the gland, PRF and PTP were observed on the same tissue section by confocal fluorescence microscopy. Merging the images taken of PRF and PTP in the mental gland tissue section reveals that PRF and PTP are colocalized within the mental gland. The co-secretion of PTP and PRF provides a biochemical signal that the mating season is ongoing. When the mental gland is stimulated PTP is released and coats the gland. By inhibiting MMP activity, PTP prevents regression of the mental gland from taking place thus preserving the gland.

Expression of rPTP

Establishing that rPTP has the same structure as PTP is necessary when using rPTP in structural studies. Preliminary evidence suggests that the structures are identical. The reverse phase chromatography ([Figure 9](#)) indicates that they both have effectively the same retention times. Expression of rPTP permits future NMR studies to determine the 3D structure of this novel TIMP-like protein. By isotopically labeling rPTP with ^{15}N ,

multiple NMR experiments can be performed, such as ^{15}N ^1H - ^{15}N HSQC, NOESY-HSQC, and ^{15}N -TOCSY-HSQC. Using this NMR data, nuclear Overhauser effects can be observed, which can then be used in conjunction with CS-Rosetta to predict an accurate model of the protein's 3D structure.^[29]

Although ^{13}C carbon labeling would be useful for determination of an NMR solution structure of a protein of this size, the approach of combining the constraints from the disulfide bonding pattern with those from the NMR data is believed to be sufficient to yield a reasonable structural prediction. ^{13}C labeling was deemed prohibitively expensive due to the high cost of ^{13}C -labeled methanol and the volume required during expression in *P. pastoris*. However, increasing the yield of rPTP in *P. pastoris* may be possible. The use of antifoaming agents in *P. pastoris* cultures has been shown to increase recombinant expression yield.^[30] The use of a *P. pastoris* clone with multiple copies of the rPTP gene may produce higher yield as well. Incremental induction using ^{13}C labeled methanol has also been shown to be an economical method for ^{13}C labeling.^[31] These techniques will be investigated provided that ^{13}C NMR experiments are necessary for greater resolution of the rPTP solution structure.

Conclusion

Using an MMP inhibition assay to test the effectiveness of PTP as having TIMP-like activity revealed that PTP can inhibit MMP-1 by approximately 15%. Using confocal fluorescence microscopy to observe the expression of PTP during the mating season indicated that when the gland is growing, PTP is not expressed. After the mental gland has matured, PTP expression levels are high. PTP is colocalized within the mental gland with PRF, which explains its presence in the pheromone extract. During courtship PTP is secreted coating the gland in PTP, which in turn inhibits MMPs from acting on the extracellular matrix. Regression of the gland is therefore prevented during the mating season. For future structural studies, a recombinant of PTP was expressed. rPTP has been shown to behave similarly in chromatography experiments. Future experiments, utilizing CD, MS and disulfide bonding pattern analyses are expected to confirm that rPTP is structurally identical to PTP.

Table 1. Relative MMP Activity with Different PTP Concentrations						
[PTP] (nM)	0	0.0225	0.225	2.25	22.5	225
hMMP-1	1.000	0.956	0.921	0.942	0.952	0.828*
hMMP-2	1.000	1.172*	1.158*	1.258*	1.260*	1.313*
hMMP-7	1.000	1.049	1.057	0.979	1.018	1.013
hMMP-8	1.000	0.956	0.959	0.935	0.930	0.905
mMMP-9	1.000	1.061	1.029	1.076	1.003	1.299*
hMMP-13	1.000	1.033	0.954	0.900	0.905	0.861

Figure 7. MMP Inhibition Assay

Results from the MMP inhibition assay using varying concentrations of PTP. At a concentration of 225nM, PTP shows statistically significant inhibition with a p-value of less than 0.05. The assay also revealed statistically significant activation of hMMP-2 by PTP. It should be noted that the purpose of this experiment was to determine if PTP possessed any TIMP-like activity and was not meant to be a quantitative determination of hMMP and PTP binding. Therefore the K_d and K_i are not directly relevant to this study.

Figure 8.

MMP inhibition assay using MMP-1 with varying concentrations of PTP. All concentrations of PTP evaluated from 7.8 to 4000 nM significantly inhibited MMP-1 by 15%. The lack of a dose-dependent response prevents an exact quantification of MMP inhibition. Although the exact level of inhibition cannot be determined precisely, the inhibition of hMMP-1 by PTP in multiple trials provides evidence that PTP may have TIMP-like activity.

Figure 9. Reverse phase chromatograms of 25ug *P. shermani* whole extract (PsWE) and 15ug of rPTP. The retention times for PTP and rPTP only differ by 0.04 minutes, which is within the margin of error for reverse phase chromatography.

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