A Novel Protein Acts as a Negative Regulator of Prophenoloxidase Activation and Melanization in the Freshwater Crayfish Pacifastacus leniusculus

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Melanization is an important immune component of the innate immune system of invertebrates and is vital for defense as well as for wound healing. In most invertebrates melanin synthesis is achieved by the prophenoloxidase-activating system, a proteolytic cascade similar to vertebrate complement. Even though melanin formation is necessary for host defense in crustaceans and insects, the process needs to be tightly regulated because of the hazard to the animal of unwanted production of quinine intermediates and melanization in places where it is not suitable. In the present study we have identified a new melanization inhibition protein (MIP) from the hemolymph of the crayfish, Pacifastacus leniusculus. Crayfish MIP has a similar function as the insect MIP molecule we recently discovered in the beetle Tenebrio molitor but interestingly has a completely different sequence. Crayfish MIP as well as Tenebrio MIP do not affect phenoloxidase activity in itself but instead interfere with the melanization reaction from quinone compounds to melanin. Importantly, crayfish MIP in contrast to Tenebrio MIP contains a fibrinogen-like domain, most similar to the substrate recognition domain of vertebrate l-ficolins. Surprisingly, asp-rich region similar to that found in ficolins that is likely to be involved in Ca2+ binding is present in crayfish MIP. However, crayfish MIP did not show any hemagglutinating activity as is common for the vertebrate ficolins. A mutant form of MIP with a deletion lacking four Asp amino acids from the Asp-rich region lost most of its activity, implicating that this part of the protein is involved in regulating the prophenoloxidase activating cascade. Overall, a new negative regulator of melanization was identified in freshwater crayfish that shows interesting parallels with proteins (i.e. ficolins) involved in vertebrate immune response.

Invertebrate animals do not have any adaptive immune systems and have to rely on innate immune systems. Several such innate systems have been described such as the coagulation system (1), melanization synthesis (2), and the production of antimicrobial peptides (3). The melanization reaction is an important component of the innate immune system of invertebrates and is essential for defense as well as for wound healing (4). In arthropods and most other invertebrates melanin synthesis is achieved by the prophenoloxidase (proPO)-activating3 system, a proteolytic cascade similar to vertebrate complement (4, 5). The proPO-activating system is initiated when microbial polysaccharides, such as lipopolysaccharides (LPS), β-1,3-glucans or peptidoglycans (PGN) are recognized by pattern recognition proteins, and the complexes formed induce activation of serine proteinase zymogens in the cascade (4, 5). The final step in this process is the conversion of proPO into the active enzyme phenoloxidase (PO). To date ~40 proPOs have been cloned and characterized, and several other constituent factors of the proPO system have recently been characterized (4, 5). Active PO oxidizes o-diphenols into quinones that are toxic to microorganisms and melanin pigments are formed that also restrict the spreading of microorganisms within the host (4, 5). Detailed studies of the molecular mechanism by which the activation is achieved has been performed in Tenebrio molitor (6), Holotrichia diomphalia (7), and Mandrauco sexta (4). In Tenebrio a complex of the peptidoglycan recognition protein Tm PGRP-SA and peptidoglycan fragments lead to the activation of proPO-activating factors, one of which (proPO-activating factor 1) in its active form is mediating cleavage of proPO into an active enzyme (6, 8). A similar mechanism of proPO activation is implicated also by research done on M. sexta (9). In crustaceans the proPO-activating system is studied in detail at the cellular and molecular level (5). In crayfish the proPO-activating system is produced in hemocytes and stored in secretory granules similar to the clotting system of horseshoe crabs (1). Upon challenge with microorganisms, the system is released into the hemolymph. Crayfish proPO is cleaved by the proPO-activating enzyme, a serine proteinase that is activated by another serine proteinase (4).
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Although melanin formation is essential for host defense in crustaceans and insects, the process needs to be tightly regulated because of the danger to the animal of unwanted production of quinone intermediates and melanization in places where it is not appropriate (10, 11). Several proteinase inhibitors, such as serpins, have been described as responsible for preventing improper activation of the proPO system (for details see Ref. 4). Pacifastatin is a high molecular weight inhibitor consisting of one light chain containing the protease inhibitors and a heavy chain that contains three transferrin lobes (12). Pacifastatin is highly efficient in inhibiting the crayfish proPO-activating enzyme (12), as is Drosophila serpin 27 in inhibiting the Drosophila proPO system and its proPO-activating enzyme (13). In addition to inhibitors of the proPO system, activation of a number of endogenous factors acting as competitive inhibitors of PO activity has been described (5). For example the so-called phenoloxidase inhibitors have been found in Musca domestica, and homologous basic lysine-rich peptides were also detected in mosquitoes and M. sexta (14, 15).

We have recently discovered a novel 43-kDa protein from the hemolymph of the beetle T. molitor (Tenebrio MIP) acting as a negative regulator of melanin synthesis (16). This protein, the target of which is presently unknown, is consumed during melanization. Interestingly, no similarity was found between Tenebrio MIP and any other known protein (16).

Here, we now report the presence of a hemolymph protein with an apparently similar function as Tenebrio MIP in the crustacean Pacifastacus leniusculus. Interestingly, this protein has no sequence similarity to Tenebrio MIP. Instead, it is similar to vertebrate ficolin and horseshoe crab Tachylectin 5 (17, 18). The crayfish MIP is very efficient in inhibiting activation of the proPO system and thus functions as an important regulatory molecule to prevent unwanted proPO activation.

EXPERIMENTAL PROCEDURES

Animals, Collection of Plasma, and Microbial Organisms—Freshwater crayfish, P. leniusculus were purchased from Torsägen (Lake Vättern, Sweden) and kept in aquaria in aerated tap water at 10 °C. Only intermolt animals were used. Hemolymph was collected by bleeding from the abdominal hemocoel through a needle (0.8 mm) into sterile tubes on ice and centrifuged at 800 × g for 10 min at 4 °C to remove the hemocytes.

The Gram-negative bacteria Hafnia alvei have earlier been isolated from P. leniusculus hemolympha and was cultured in LB broth. For fresh cultures the bacteria were grown with shaking at 260 rpm overnight at 37 °C and then diluted 1:100 and further cultured until the density reached OD600 of ≈0.5. Bacteria H. alvei were injected into the base of the fourth walking leg as earlier described (19).

Hemocyte Lysate Supernatant—Hemocyte lysate supernatant (HLS) was prepared by collecting hemolymph from 8 crayfishes in bleeding buffer (10 mM sodium cacodylate, 250 mM sucrose, pH 7.0). The hemocytes were spun down by centrifugation at 800 × g for 10 min at 4 °C and then homogenized in 10 mM sodium cacodylate, 5 mM CaCl2, pH 7.0. The homogenate was centrifuged at 25 000 × g for 20 min at 4 °C, and the supernatant was adjusted to a protein content of ~1 mg/ml, kept on ice, and used as HLS within 1 h.

Induction of proPO Activation and Assay of PO Activity—To confirm the involvement of crayfish MIP in the proPO system, 25 μl of HLS (1 mg/ml) was preincubated with 25 μl of MIP (wild type or MIP(–D)), a mutant protein lacking the tetra-aspartic acid stretch, at 0.5–1 μg) or buffer for the control for 10 min at 20 °C. These mixtures were incubated with 25 μg of LPS-PGN (Sigma L3129 from Escherichia coli 0127:B8) and 25 μl of 3,4-dihydroxy-L-phenylalanine (L-DOPA, 3 mg/ml) for 5–20 min at 20 °C. For analysis of the effect of MIP on PO activity, HLS was preincubated with LPS-PGN for 25 min at 20 °C to fully activate proPO prior to the addition of MIP. Phenoloxidase activity was measured as the oxidation of L-DOPA at 490 nm and presented as the means ± standard deviation from four independent experiments. In some experiments the phenoloxidase inhibitor phenylthiourea (PTU) was preincubated with HLS for 5 min at 20 °C.

Measurement of Proteinase Activity—To determine whether any activating proteinase was affected by MIP, LPS-PGN-activated amidase activity of HLS was assayed as the hydrolyzing activity toward the chromogenic peptide S-2222 (Suc-Ile-Gly (ypPip) Gly-Arg-pNA; Chromogenix). Briefly, 25 μl of HLS was incubated with 25 μg of LPS-PGN and 100 μl of 100 mM Tris-HCl, pH 8.0, and 25 μl of 2 mM S-2222 at 30 °C for 30 min, and then the reaction was terminated by the addition of 50 μl of 50% acetic acid, and the absorbance at 405 nm was determined. The effect of MIP was determined by preincubation of MIP (0.5–1 μg) or buffer for the controls for 10 min at 20 °C prior to the addition of LPS.

Detection of Crayfish MIP in Plasma and Determination of the Amino Acid Sequences—The proteins in plasma were precipitated with acetone and subjected to 12.5% SDS-PAGE or two-dimensional gel electrophoresis under reducing conditions. First-dimensional separation was performed according to pl, and second-dimensional separation was done according to molecular weight. The IPG strips (7 cm length, pl range between 3 and 11, nonlinear; GE Healthcare) were rehydrated with rehydration solution including 80 μg of protein extracted before, for 12 h or overnight at 20 °C. Using the IPGphor system (GE Healthcare), isoelectric focusing was performed at a total of 45.5 kVh at 20 °C. The cytochrome c was reduced and carboxymethylated, whereas the proteins were equilibrated in the two-dimensional loading buffer (glycerol, SDS, urea) supplemented with 1% dithiothreitol for 15 min at 20 °C, followed by 2.5% iodoacetamide in fresh equilibration buffer for an additional 15 min at 20 °C. After equilibration, the IPG strips were applied onto a 7-cm acrylamide gel (12.5%). SDS-PAGE was performed at 30 mA/gel for 50–60 min at 20 °C. All of the electrophoretic procedures were performed at room temperature. One two-dimensional gel was stained with Coomassie Brilliant Blue R-250, and the other gel was transferred to a polyvinylidene difluoride membrane for Western blot. An affinity-purified antibody against Tenebrio MIP (16) was used for Western blot analysis. The proteins were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane, blocked by immersion in 5% skimmed milk solution for 1 h. The membrane was then transferred to TBS (10

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*a* I. Söderhäll, unpublished observation.
were precipitated with acetone and analyzed by Western blot after SDS-PAGE under reducing conditions as described above.

cDNA Cloning and Nucleotide Sequencing of 43-kDa Crayfish MIP—Hepatopancreas total RNAs were extracted using GenElute™ mammalian total RNA miniprep kit (Sigma) and followed by RNase-free DNase I (Ambion, Austin, TX) treatment. cDNA was synthesized with ThermoScript (Invitrogen). Several sets of gene-degenerate primers based on the MS sequence results (VMMEDFDANK) were designed for 5'-rapid amplification of cDNA ends (RACE) and 3'-RACE. 5'-RACE was performed according to the manufacturer’s protocol (Invitrogen). Oligo(dT) was used to synthesize first strand cDNA. After poly(C) tail were assembled, PCR amplification was performed using the MIP-5R1 (5'-TTTGCCTCRAARTCTTCATC-3') and abridged anchor primer followed by nest PCR amplification with MIP-5R2 (5'-TCTTACGAGCTCATAC-3') and abridged universal amplification primer (AUAP) using the recommended conditions. For 3' RACE, oligo(dT)-adapter was used to synthesize first strand cDNA. An initial amplification by PCR was carried out with primer MIP-3R1 (5'-GGGAGAGAGAGAGGCTTT-3') and oligo(dT) adapter. The nested PCR was performed with primer MIP-3R2 (5'-GGGTTCACGAGCTCATAC-3'). Amplified fragments were cloned into PCR 2.1-TOPO TA cloning vector (Invitrogen) and sequenced.

**Determination of MIP mRNA Localization**—Total RNA extraction from different tissues (hepatopancreas, eyestalk, hemocytes, nerve tissue, heart, muscle, intestine, and hematopoietic tissue cells) was performed using TRIzol LS reagent (Invitrogen), followed by chloroform extraction and ethanol precipitation of the aqueous phase. Total RNA was treated by RNase-free DNase I (Ambion, Austin, TX) treatment. cDNA was synthesized with ThermoScript (Invitrogen) followed by PCR using primers specific for MIP (GenBank™ accession number EX571686). Crayfish 40 S ribosomal (R40s; GenBank™ accession number CF542417) primers were used in all PCR experiments as control. The primers used were as follows: MIP 217–5', 5'-CCACTCACCTCAGCCGACAC-3'; 5'-TTCTCCCATGAGTACGTCTCAGCT-3'; crayfish 40 S ribosomal protein gene 5+, 5'-CCAGGACCCCCAAATCTCTTAG-3'; and 364–, 5'-GAAAACTGCCACAGCC-3'. For detection of MIP in the RNA interference experiment MIP 1+, 5'-TACGACCTTCGATCTTACTCTCA-3' were used. The PCR program was as
follows: 94 °C for 2 min, followed
by 30 cycles of 94 °C for 30 s, 58 °C
for 30 s, and 72 °C for 40 s for the
MIP gene and 28 cycles for the
R40s. The PCR products were ana-
lyzed on a 2% agarose gel stained
with ethidium bromide.

Expression and Purification of
Recombinant Crayfish MIP—
The construction of the recombi-
nant baculovirus vector and the
expression of the recombinant
MIP (rMIP) were performed
according to manufacturer’s in-
structions (Invitrogen). The cDNA
encoding the mature MIP was sub-
cloned into pFastBac1 vector
(Invitrogen) using BamHI and Sall
enzyme sites. The recombinant
virus for MIP expression was gen-
erated according to the manufac-
turer’s instruction manual (Bac-
to-Bac Baculovirus expression
system; Invitrogen). The recombi-
nant virus was amplified using
Spodoptera frugiperda 9 (Sf9;
Invitrogen) cells in SF-900II
serum-free medium (Invitrogen)
at 27 °C. To produce the protein,
Sf9 cells were grown in Sf-900II
serum-free medium (Invitrogen)
in a 75-cm² tissue culture flask.
The cells were infected with a cell
density of 2 × 10⁶ cells/ml at a
multiplicity of infection of 10 and
were incubated for 3–4 days. The
supernatant was collected by cen-
trifuging at 500 × g for 10 min.
After discarding the pellets, the
supernatant containing MIP pro-
tein was desalted by using a PD-10
desalting column (code 17-0851-
01; Amersham Biosciences) and
applied to a 1-ml HiTrap Q HP col-
umn, pre-equilibrated with 20
mM Tris-HCl buffer, pH 8.0, and
then this column was washed with
the same buffer until no material
appeared in the effluent and then
gradient eluted with 20 mM Tris-
HCl, 1 M NaCl, pH 8.0 (100 ml,
0–50%) at a flow rate of 60 ml/h.
Fractions of 1 ml were collected.
The purified protein was subjected
to 12.5% SDS-PAGE and stained
with Coomassie Brilliant Blue.
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Site-directed Mutagenesis—The cDNA encoding the mature crayfish MIP was subcloned into pFastBac1 vector (Invitrogen), and this plasmid was used for mutagenesis. Site-directed mutagenesis was done using the QuikChange site-directed mutagenesis kit (Stratagene). The mutants were obtained by deleting the four aspartic acids in the Asp-rich region of the cDNA clone: MIP(−D4) 858−: 5′-TTTTCCCTACGCA-CAAGAACAGTGTGAACTGTCT-3′; and MIP(−D4) 858−: 5′-GAGCGTATCCTTGTTCTGCGTGA-GTGAAAC-3′. The PCR was done as follows: 95 °C for 30 s, and 18 cycles of 95 °C for 30 s, 55 °C for 1 min, final extension at 68 °C, 6 min. The nicked vector DNA containing the desired mutations was then transformed into XL-Blue supercompetent cells, and then mutant MIP(−D4) plasmids were purified and sequenced to verify the mutated sequence. The generation of the recombinant virus for crayfish MIP(−D4) expression and the purification of recombinant crayfish MIP(−D4) is similar to the methods used for crayfish MIP.

Hemagglutinating Activity Assay—Hemagglutinating activity toward human erythrocytes type A, B or O of rMIP was determined in assay buffer containing 5 mM CaCl2 as described in Ref. 20.

RNA Interference Experiments—Oligonucleotide primers including 17 promoter sequences (italics) at the 5′ end were designed to amplify a 776-bp region of the P. leniusculus MIP gene: 217+5′-TATACGACTCATAFGGCGGACATCCTCA-GGCCGACAC-3′; 993−5′-TATACGACTCATAFGGCCGAGGGCCCGCTACTCGTTA-3′. The PCR was done as follows: 95 °C for 30 s, and 15 cycles of 95 °C for 30 s, 55 °C for 1 min, final extension at 68 °C, 6 min. The PCR products were purified by gel extraction and sequenced to verify the mutated sequence. The generation of the recombinant virus for crayfish MIP expression and the purification of recombinant crayfish MIP is similar to the methods used for crayfish MIP.

Cloning and Characterization of the cDNA for This Crayfish Protein—To determine the amino acid sequence of this crayfish protein, we designed degenerate primers to the peptide VVMEDFDANK and performed 5′-RACE and 3′-RACE using cDNA synthesized from hepatopancreas RNA. When we used hemocyte cDNA no transcript could be detected. However, we obtained a cDNA of 1764 base pairs from hepatopancreas, and the deduced amino acid sequence of the open reading frame of this cDNA is shown in Fig. 2A. This cDNA encodes a protein consisting of 326 amino acid residues with a signal sequence of 26 residues. The deduced protein sequence was confirmed to have identical theoretical tandem MS spectra to the protein spot shown in Fig. 1B. The crayfish protein contains three putative N-linked glycosylation sites in the deduced amino acid sequence (open circles in Fig. 2A), and the first at position 30 (NPSI) is likely to have carbohydrates bound according to the tandem MS result.

Surprisingly the sequence of this Pacifastacus 43-kDa protein is totally different from the Tm MIP, and instead the crayfish 43-kDa protein shows a significant sequence similarity in its C terminus to vertebrate fibrinogens. A homology search revealed also a certain similarity with the fibrinogen-related domains (FReD) of vertebrate fibrinogens, but the collagenous domain found in these proteins is missing in the corresponding region of the crayfish 43-kDa protein (supplemental Fig. S1). Pairwise comparisons of the FReDs showed that the crayfish...
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43-kDa protein showed higher sequence similarities with vertebrate ficolins as compared with horseshoe crab tachylectins (TL) 5A and 5B, although the molecular size and missing collagenous domain is shared with the tachylectins. TL-5A and TL-5B (TLs-5) contain six and seven cysteine residues, respectively, and two/three of these are supposed to be involved in interchain disulfide linkages. However, the crayfish 43-kDa protein is apparently present in plasma as monomers because a Western blot under nonreducing conditions did only show one band at ~43–45 kDa (Fig. 1A) and contains solely four cysteines corresponding to the cysteine residues that in tachylectins and ficolins are involved in intrachain disulfide linkages (Fig. 2B). The TLs-5s have efficient hemagglutinating activity and are easily purified by binding to N-acetyl group-immobilized resins as are l-ficolins (21). In contrast, the crayfish 43-kDa protein does not bind to this resin and has no hemagglutinating activity toward A, B, or O type human erythrocytes (a concentration range of 0.005–25 μg/ml was tested).

When comparing the amino acid sequence of the crayfish 43-kDa protein with that of Tm MIP, the similarities were few. To explain the similar antigenicity of these two proteins, we compared their antigenic index plot using the MacVector 7.0 software. The highest index was obtained for the Asp-rich regions that are common to both proteins. As shown in Fig. 2C, Tm MIP contains a region in its central part with 11 Asp residues, whereas the crayfish 43-kDa protein has an Asp-rich region containing five Asp residues with four Asp in one row. This region is probably recognized by the Tm MIP antibody.

Expression of the Crayfish 43-kDa Protein—We cloned the first transcript using RNA extracts from hepatopancreas, whereas no mRNA encoding this protein was found in hemocytes. After obtaining the full sequence, we then analyzed the expression pattern in different tissues by reverse transcription-PCR. As shown in Fig. 3, the transcript for the crayfish 43-kDa protein was detected at fairly low level in hepatopancreas and eyestalk, whereas high expression occurred in nerve tissue, heart, and intestine. Hemocytes and hematopoietic tissue cells did not express this transcript.

Recombinant Pacifastacus MIP Inhibits Melanization in Vitro—Because of the similar antigenicity of the crayfish 43-kDa protein and Tm MIP, we decided to explore the relationship between the 43-kDa protein and the proPO-activating system in crayfish. Accordingly, we produced the recombinant protein and purified it to homogeneity (supplemental Fig. S2). From these results (supplemental Fig. S2), it is also evident that this recombinant protein was detected by the Tm MIP antibody. Then we added the recombinant protein to a fresh preparation of HLS containing an inactive proPO system. As shown in Fig. 4A, this recombinant protein could inhibit LPS-PGN or β-1,3-glucan induced PO activity in a dose-dependent manner assayed with l-DOPA as substrate. When proPO was activated prior to incubation with the recombinant protein, no such inhibition was achieved (Fig. 4B).

To investigate whether the antigenic Asp-rich region is important for the function of the crayfish 43-kDa protein, site-directed mutagenesis was performed by deleting the four-Asp amino acids in the recombinant protein. As anticipated, this mutated protein showed significantly decreased inhibitory activity compared with when the aspartic acid motif was intact (Fig. 4C).

Furthermore, even if the enzyme catalyzed oxidation of l-DOPA by the active PO was unaffected by the recombinant 43-kDa protein, the following nonenzymatic autocatalytic oxidation leading to melanin formation was completely blocked in its presence (Fig. 5A). Because we found that the crayfish 43-kDa pro-
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The protein also appears to be involved in regulating the proPO system and melanization, we decided to name it *P. leniusculus* (Pl) MIP. Our results suggest that *Pl* MIP functions by two different mechanisms. One is to inhibit proPO activation, and the other is to block or delay melanin formation, once PO is activated. We therefore decided to investigate the influence of recombinant *Pl* MIP on LPS-PGN-induced proteolytic activity, using the commercial substrate S-2222 that we have shown is a good substrate for MIP. The concentration of rMIP chosen in these *in vitro* inhibitory experiments was in physiologically relevant concentrations. As shown in Fig. 5B, LPS-PGN could induce proteolytic activity in an inactive HLS as compared with the control without LPS-PGN. If r-Pl MIP was preincubated with the inactive HLS prior to the addition of LPS-PGN, a delay in the appearance of proteolytic activity was evident (Fig. 5B). Because *Ca*²⁺ is known to be a prerequisite for proPO activation, we also performed experiments where *Ca*²⁺ was added to the mixture to test whether the inhibitory effect of r-Pl MIP was a result of *Ca*²⁺ entrapment. However, no effect on the inhibitory activity was achieved by the addition of *Ca*²⁺. This indicates that rMIP does not affect the activating mechanism by some other mechanism.

_Tenebrio_—MIP was detected because of its disappearance after activation of the proPO system during melanization of the hemolymph (10), indicating that the protein was degraded during melanin formation. We decided to examine whether *Pl* MIP disappeared in a similar way during melanin synthesis. Therefore, we induced activation of proPO by LPS-PGN in the presence or absence of the PO inhibitor PTU after preincubation with r-*Pl* MIP. As we expected, incubation in the presence of PTU did not result in oxidation of L-DOPA, and r-*Pl* MIP was unaffected (Fig. 5C). When PTU was absent from the reaction mixture, oxidation of L-DOPA proceeded, and then r-*Pl* MIP disappeared completely from the reaction mixture (Fig. 5C).

_Crayfish MIP Is Affecting Melanin Synthesis in Vitro as well as in Vivo—_*To get information about the *in vivo* function of *Pl* MIP, we performed RNA interference experiments in live crayfish using a method we have successfully used to silence proPO as well as the proPO-activating enzyme inhibitor pacifastin (13). However, we were unable to silence MIP expression in live crayfish, probably because *Pl* MIP is transcribed at a high level in many different tissues. Therefore, we carried out further experiments to reveal information about the *in vivo* function of *Pl* MIP.

First we separated granular cells and incubated these cells in L15 medium containing plasma immunodepleted with antibodies against *Pl* MIP, the medium was heavily melanized, and melanin particles were spread all over the culture dish (Fig. 6B). These experiments clearly indicate that *Pl* MIP is involved in regulating melanin formation in crayfish. Furthermore, when recombinant MIP protein was added to cells treated with anti-MIP-immunodepleted plasma, melanization was clearly inhibited (Fig. 6C). To further establish whether *Pl* MIP is related to melanin synthesis of the hemolymph *in vivo*, we induced melanization in crayfish by injecting the Gram-negative bacterium _Hafnia alvei_, known to induce melanin synthesis in crayfish. In Fig. 6D it is shown that injection of *H. alvei* results in a heavy melanization of the hemolymph, and simultaneously a decrease in hemolymph *Pl* MIP concentration was clearly visible (Fig. 6E). On the other hand injection with the highly pathogenic bacterium _Aeromonas hydrophila_ neither caused a change in *Pl* MIP content of the hemolymph nor induced melanization (Fig. 6, D and E).

Structure of the Protein and Calcium Binding—_The sequence similarity between *Pacificatus* MIP and the recognition domain of human L-ficolin was used to build a homology model.

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5I. Söderhäll, unpublished results.
of the *Pacifastacus* MIP three-dimensional structure (Fig. 7). The model suggests that *Pacifastacus* MIP is an alpha/beta protein stabilized by two cysteine bridges. The binding site for Ca$^{2+}$ ions as described in the *ficolin* structure and similar to invertebrate lectin tachylectin 5A (13) is rather well conserved in *Pl* MIP. Our experiments using a mutant lacking this putative Ca$^{2+}$-binding site showed limited inhibitory activity (Fig. 4C), suggesting a role for this site in the function of *Pl* MIP. Crucially, two Asp residues that form specific contacts to Ca$^{2+}$ ions through their side chains carboxyl groups in the ficolin structure appear in the model, too. Interestingly, the putative Ca$^{2+}$-binding site of *Pl* MIP is longer by one amino acid (Lys) compared with ficolin. Nevertheless, this should not prevent Ca$^{2+}$ ions from binding because the remaining interactions between Ca$^{2+}$ ions and ficolin are mediated through interactions with the carboxyl groups of the main chain. Therefore, the insertion of an amino acid into the site will not influence the availability of a carboxyl main chain group for ion coordination. The amino acids described as defining the specificity of substrate binding in *ficolin* structure are not conserved in the suggested model of *Pl* MIP; therefore different ligands might be expected.

**DISCUSSION**

In the present study we have identified a novel protein named *Pacifastacus* MIP from crayfish hemolymph that interferes with the melanization reaction in this animal. We recently described for the first time a protein with a similar function but with a different structure in an insect, *T. molitor* (10). This is therefore the second protein found to act as a negative regulator of melanin formation in invertebrates. A number of nonproteinaceous factors acting as inhibitors of PO activity have earlier been described (3, 23), and phenoloxidase inhibitors peptides are described in several insect species (24, 25), but all are shown to inhibit the PO activity. Other proteins affecting melanization are known as proteinase inhibitors that will inhibit one or several of the proteinases that are components of the proPO system, and therefore they will inhibit proPO activation. Among these latter inhibitors is a large molecular weight inhibitor, pacifastin, from *P. leniusculus* acting as a potent inhibitor of the proPO-activating protease, and *Drosophila* Spn27, a small serpin supposed to inhibit the proPO-activating enzyme (14, 26). In contrast, *Pl* MIP and *Tm* MIP do not affect PO activity in itself but
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instead interfere with the melanization reaction from quinone compounds to melanin. However, in contrast to Tm MIP, the crayfish protein was also shown to delay the induced protease activity that is responsible for proPO activation, but it did not inhibit an already LPS-PGN-activated protease, suggesting that proPO activation is affected by this protein in addition to its effect of inhibiting the following melanin formation from L-DOPA. PI MIP was found to be expressed in many different tissues, but not in the hemocytes or in the hematopoietic tissue, and the highest expression was detected in heart, nerve tissue, and intestine. Unfortunately, we were unable to silence the expression of PI MIP, and therefore no detailed in vivo functional studies of this protein could be performed. However, we earlier identified a bacterium from crayfish hemolymph as H. alvei (previously isolated from melanized hemolymph) that is inducing melanization of the hemolymph after injection. When plasma was analyzed for the presence of PI MIP after injection of this bacterium, melanization was induced, and the MIP protein was found to be absent (Fig. 6E), indicating that PI MIP has a role as a negative regulator of melanin formation in vivo as well.

Interestingly, we have been able to isolate a protein from crayfish hemolymph with an apparently similar function as Tm MIP by using heterologous antibodies, although these two proteins are completely different when their amino acid sequence are compared. However, the most probable common antigenic surface of Tenebrio and Pacifastacus MIP is the long Asp-rich region in Tm MIP and the shorter Asp stretch found in PI MIP. These Asp-rich regions are likely to be involved in Ca\(^{2+}\) binding of these proteins. Interestingly, the Asp-rich region in PI MIP is surrounded by cleavage sites for trypsin-like proteases, and the sequence is highly similar to the acidic tetra-aspartate sequence of the activation peptide of human trypsinogen (27). The conserved tetra-Asp in trypsinogen is required for enteropeptidase recognition and cleavage and the activation peptide functions as an effective inhibitor of trypsin autoactivation (28). The pro-PO-activating cascade involves the activation of several proteases with trypsin-like activity (3). The ability of MIP in inhibiting the activating PO activating cascade was significantly decreased, after its Asp-rich region was deleted (Fig. 4C). This result proves that the tetra-Asp stretch in crayfish MIP is involved in regulating the activating cascade.

Although Tm MIP so far does not show any significant similarity with any known protein, PI MIP in contrast contains a FReD, most similar to the recognition sites of vertebrate ficolins (14). Lack of the collagenous domain and the molecular size (43 kDa) of PI MIP is similar to that of TL5s in horseshoe crabs (21), but PI MIP did not show any hemagglutinating activity and is not likely to have lectin activity as the TL5s. Human 1-ficolin was then used as a template for building a homology model of the PI MIP three-dimensional structure, and the binding site for Ca\(^{2+}\) was found to be highly conserved. The putative Ca\(^{2+}\) binding site of PI MIP is longer than that of ficolins, and the first two Asp-residues of this site are flanked by another two Asp forming a tetra-Asp stretch that is not found in ficolins or other fibrinogen-related domains. These sequence differences may indicate a different ligand in PI MIP.

In summary we have isolated a protein, crayfish MIP, with a similar function as Tm MIP, but with a totally different molecular structure. The PI MIP is most likely an important regulator of the proPO system and will keep the proPO system in a nonactive form until specific inducers such as pathogen-associated molecular patterns or microorganisms are present. Then PI MIP as well as Tm MIP (10) are degraded, which then will allow activation of the proPO system and melanization. The structural similarities of PI MIP with ficolins known as activators of vertebrate complement are also interesting and indicates parallels in the regulation between proteolytic cascades involved in defense in vertebrates and invertebrates.

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