MOLECULAR INTERACTIONS AMONG PAP-1, SPHS AND PROPO IN ACTIVATION OF PROPHENOLOXIDASE

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MOLECULAR INTERACTIONS AMONG PAP-1, SPHS AND PROPO IN ACTIVATION OF PROPHENOLOXIDASE

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CHAPTER I

INTRODUCTION

Immunity is the ability of a multicellular organism to defend itself against invading pathogens. There are two types of immunity mainly. Acquired immunity involves production of antibodies, T-cells, B-cells and takes 2-3 weeks to develop. Found in vertebrates only, acquired immunity has specificity, adaptability, and memory. On the other hand, innate immunity is fast but has no memory and is less specific. It is found in all animals, including vertebrates and invertebrates.

Insects account for more than 70% of the animal species on the earth. One of the reasons for their evolutionary success is the efficient defense system. Insects rely solely on innate immunity to defend against microbial infections. In-depth study of innate immunity in biological models, such as fruit fly, silkworm and tobacco hornworm, may help us to better understand the role of innate immunity in human defense system. It may also help us in undertanding the evolution of innate immune system from invertebrates to vertebrates, since conserved immune pathways exist in *Drosophila melanogaster* and *Homo sapien.*

Many parasites evade insect defense responses and use the hosts as vectors for disease transmission. Each year, malaria claims more than a million deaths in the world. A strain of the mosquito, *Anopheles gambiae*, which is refractory to infection by *Plasmodium cynomolgi* encapsulate ookinetes of the malaria parasite in a melanin coat,

whereas susceptible strains do not (Collins et al., 1986). Biochemical analysis of melanotic encapsulation, a phenoloxidase-mediated immune response, may lead to new strategies for disrupting disease transmission.

Insect defense responses can be triggered upon binding of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Gillespie et al., 1997; Lavine et al., 2002). A variety of PRRs, present in the insect hemolymph or on the surface of immune cells, specifically recognize peptidoglycan, lipopolysaccharide (LPS), or β-1,3-glucans – cell wall components commonly found in bacteria/fungi but are absent in host tissues or cells. Pattern recognition initiates phagocytosis, nodulation, encapsulation, melanization, and synthesis of antimicrobial peptides to sequester and kill the invading pathogens.

Melanization (Sugumaran, 1996; Nappi and Vass, 2001) is catalyzed by phenoloxidase (PO) which converts monophenols to *o*-diphenols and oxidizes *o*-diphenols to *o*-quinones (Mason, 1965). Due to potential cytotoxicity of quinones and other reactive intermediates, PO activity and its proteolytic activation from prophenoloxidase (proPO) have to be strictly regulated as a local reaction against nonself. The proPO activation is mediated by a largely unknown proteinase cascade, analogous to the coagulation system and complement activation pathway in human plasma.

The proPO activating proteinase/enzyme/factor (PAP, PPAE, or PPAF) has been isolated and cloned from *Manduca sexta* (Jiang et al., 1998, 2003a, 2003b), *Bombyx mori* (Satoh et al., 1999), *Holotrichia diomphalia* (Lee et al., 1998a), and *Pacifastacus leniusculus* (Wang et al., 2001c). In the silkworm, PPAE itself converts inactive proPO to active PO. However, in *H. diomphalia* (Lee et al., 1998a) and *M. sexta*, serine

proteinase homologs (SPHs) are also needed for proPO activation by PAP or PPAF. SPHs are similar in sequence to serine proteinases, but they lack proteolytic activity since the active site Ser is replaced by Gly (Yu et al., 2003). *M. sexta* SPH-1 and SPH-2 bind to immunolectin-2, a C-type lectin which interacts with LPS of Gram-negative bacteria. Therefore, SPHs may localize proPO activation to the site of pathogen infection.

In our laboratory, we previously isolated and cloned three PAPs from the tobacco hornworm, *M. sexta* (Jiang et al., 1998; Jiang et al., 2003a; Jiang et al., 2003b). PAP-1 (from cuticle) has one clip domain and one catalytic domain, whereas PAP-2 and PAP-3 (from hemolymph) contain two clip domains and one catalytic domain. All three PAPs require SPHs, as a cofactor perhaps, for generating active PO (Yu et al., 2003). Similar to the PAPs, *M. sexta* SPHs have a regulatory clip domain at the amino terminus.

ProPO activation is negatively regulated by serine proteinase inhibitors of the serpin superfamily (Kanost et al., 2001). Serpins form an acyl-enzyme complex with the active site Ser of PAPs and block their proteolytic activity. It is proposed that this process prevents the diffusion of PAPs away from the site of infection.

So far, it is not clear what role PAPs and (especially) SPHs play during proPO activation. The specific aims of my research are to purify and characterize *M. sexta* PAP-1 from the cuticles and examine the roles of proteolytic cleavage and SPHs in the activation reaction.

CHAPTER II

LITERATURE REVIEW

Insects have several well-developed defense mechanisms that are essential for fighting against microorganisms. Firstly, cuticle and peritrophic membrane serve as a physical barrier to prevent the entry of pathogen (Sugumaran, 1996). When a pathogen breaches the physical barrier, it may encounter and trigger the host innate immunity, which is mediated by hemocytes, fat body, and plasma proteins. Hemocytes undergo phagocytosis, nodulation, and encapsulation to eliminate and entrap the pathogen (Gillespie et al., 1997; Hoffmann and Reichhart, 2002). The defense response also involves induced synthesis of antimicrobial peptides and PO-mediated melanization.

Cuticle

In insects, cuticle is the outermost layer secreted by epidermis and it also lines the stomodeum, proctodeum, and trachea thereby protecting not only external surface but also internal surfaces that are accessible to foreign invaders. Sclerotized cuticle is an effective physical barrier against wounds and microbial invasion. Apart from that, cuticle also prevents water loss and provides shape and strength to the insect. Cuticles, mainly composed of chitin (polymers of β-1,4-*N*-acetylglucosamine) and proteins, are reinforced by sclerotization (*i.e.* crosslinking of proteins and chitins by highly reactive quinones). Melanization results from polymerization of quinones (Hopkins et al., 1992).

Cellular responses

Cellular responses, mediated by circulating hemocytes, include phagocytosis, nodulation, and encapsulation. Phagocytosis is the process of ingestion and digestion of small foreign particles. Being phagocytic cells, granulocytes and plasmatocytes can engulf bacteria. In case pathogens (*e.g.* parasitoid eggs and nematodes) are too large to ingest, these hemocytes can form multilayered capsules to immobilize the invading organisms. When bacteria or fungi are in large numbers, hemocytes as well as plasma proteins can aggregate them and result in the formation of nodules. Oenocytoids produce proPO, and the active form of proPO causes melanization to entrap and kill the invading pathogen (Ashida and Brey., 1998).

Humoral responses

Humoral responses include synthesis of antimicrobial peptides in response to a pathogen invasion. These peptides are mainly synthesized in fat body and secreted into the hemolymph to kill the pathogen. Humoral responses also include proPO activation and hemolymph coagulation reaction.

Proteolytic activation of proPO is mediated by a serine proteinase cascade, which eventually converts inactive proPO to active PO. Active PO produces quinones which polymerize to form melanin. As quinones and its intermediates are probably harmful to pathogens as well as host cells, this pathway is strictly regulated by various mechanisms. Our knowledge on proPO activation and regulation is discussed in detail below.

Pattern recognition receptors (PRRs)

As the essential first step, hosts have to recognize pathogen as an intruder before a defense response is initiated. In insects, PRRs in hemolymph or on hemocyte surface are responsible for such nonself recognition. They bind to the conserved molecular patterns on the outer layer of microbial cells, named pathogen-associated molecule patterns (PAMPs) (Yu et al., 2002b; Janeway and Medzhitov, 2002). PAMPs include LPS of Gram-negative bacteria, peptidoglycan of Gram-positive bacteria, as well as glucans and mannans of fungi. A number of studies have shown the existence of insect PRRs such as LPS-binding proteins, β-1,3-glucan recognition proteins (βGRPs), and peptidoglycan recognition proteins (PGRPs).

Insect PGRPs are constitutively expressed at low levels and increase in expression levels upon challenge with bacteria. They play an important role in the proPO activation pathway (Yoshida et al., 1996; Lee et al., 2004). PGRP sequences are conserved from insects to mammals. Four PGRPs have been found in human (Kang et al., 1998). In *Drosophila*, PGRP-LC is involved in the Imd pathway (Ramet et al., 2002; Werner et al., 2003).

M. sexta immunolectin-2 (IML-2), a C-type lectin that binds to LPS, associates with serine proteinase homologs (SPHs) to perhaps localize proPO activation and PO activity near the pathogen infection site (Yu et al., 2003). IML-2 is present at a low level, and its expression is up-regulated in response to Gram-negative bacteria (Yu et al., 1999; Yu et al., 2000; Yu et al., 2001). As PRRs, C-type lectins in mammals also recognize microorganisms and trigger immune responses.

Similarly, βGBPs in *B. mori*, *M. sexta*, *H. diomphalia*, and *P. leniusculus* play a role in the proPO activation system (Ma et al., 2000; Ochiai et al., 2000; Zhang et al., 2003; Jiang et al., 2004).

Serine proteinases (SPs)

Most SPs are extracellular proteins found both in vertebrates and invertebrates. They have diverse roles in biological processes such as digestion, blood coagulation, reproduction, and development. SPs contain a catalytic triad, consisting of His^{57}, Asp^{102}, and Ser^{195} (chymotrypsin numbering). The active site Ser^{195} is involved in acyl transfer, His^{57} acts as a proton acceptor, and Asp^{102} stabilizes His^{57} (Gorman and Paskewitz, 2001). Many SPs work in a cascade mode, *i.e.* one proteinase cleaves and activates another proteinase which activates the third one. Human blood coagulation system is a good example of such SP cascade. In arthropods, SP pathways are responsible for horseshoe crab hemolymph coagulation (Iwanaga et al., 1998), proPO activation (Kanost, et al, 2004), and proteolytic processing of cytokines (*e.g.* spätzle and plasmatocyte-spreading peptides) (Hoffmann and Reichhart, 2002) (Wang et al., 1999).

Many immune-related SPs contain a regulatory clip domain at the amino terminus and a catalytic domain at the carboxyl terminus. These proteins are stored as zymogens and activated upon cleavage at a specific peptide bond. In these active SPs, the clip domain remains attached to the catalytic domain via an interchain disulfide bond. Clip domain, a compact structure stabilized by three disulfide bridges, is commonly found in arthropod SPs and SPHs. The function of clip domains is still unknown. It may mask the active site of its zymogen and on activation the clip domain unfolds and exposes the

catalytic site. Clip domain may interact with a microbial surface, an upstream SP, a downstream substrate in the SP cascade (Jiang and Kanost, 2000).

Prophenoloxidase activating enzymes (PPAEs)

PPAE is the last SP in the proPO activation pathway. They directly cleave proPO to generate PO. Like other SPs, PPAEs are stored as inactive precursors and activated by limited proteolysis. Different terminologies are used for the same enzymes isolated from different arthropod species.

B. mori PPAE contains two clip domains and one catalytic domain that are covalently linked via a disulfide bond (Satoh et al., 1999). The proenzyme of PPAE, proPPAE, is converted to active proteinase by a proteolytic cleavage between Lys^{152} and Ile^{153}. The silkworm PPAE is 36.9%, 34.5%, and 35% identical in sequence to *D. melanogaster* easter, *M. sexta* PAP-1, and *H. diomphalia* PPAF-I, respectively. PPAE mRNA is detected in integuments, hemocyte, and salivary gland, but not in fat body (Satoh et al., 1999).

In *H. diomphalia*, PPAF-I has been purified from the larval hemolymph (Lee et al., 1998a). Based on its cDNA sequence, the calculated molecular mass of proPPAF-I is 40 kDa and the beetle SP is 42.9% similar in sequence to *Drosophila* easter.

In *M. sexta*, three SPs have been isolated from the prepupae. They are PAP-1 from integument (Jiang et al., 1998), PAP-2 (Jiang et al., 2003a) and PAP-3 (Jiang et al., 2003b) from hemolymph. PAP-1 is similar to *Drosophila* easter which contains a single clip domain and a catalytic domain linked by a disulfide bond. Its molecular mass is 44 kDa. PAP-1 cDNA was cloned from a hemocyte cDNA library. The inactive proPAP-1

becomes active PAP-1 by a cleavage between Arg^{127} and Ile^{128} of proPAP-1. Recombinant proPAP-1 and proPAP-2 have been successfully expressed in baculovirus (Wang et al., 2001; Ji et al., 2003). Supplementation of hemolymph with proPAP-1 or proPAP-2 enhanced proPO activation after *M. luteus* was added to the induced larval hemolymph.

Similar to the silkworm PPAE, *M. sexta* PAP-2 and PAP-3 have two clip domains at the amino terminus and one catalytic domain at the carboxyl terminus. The molecular masses of the PAP-2 clip domains and catalytic domain are 25 and 35 kDa, respectively. The activation site for PAP-2 lies between Lys^{153} and Ile^{154}. PAP-3 is a 44 kDa protein, whose activation site lies between Lys^{146} and Ile^{147}.

The mRNAs of PAP-1 and PAP-3 are present at low levels in fat body of the naïve larvae. Upon bacterial challenge, their levels were significantly increased. All these three PAPs are synthesized as inactive zymogens and become active via limited proteolysis by some unknown proteinases. Active PAPs cut the proPO at Arg^{51}, but the cleaved proPO is not active as a PO. Serine proteinase homologs (SPHs), which may act as cofactor of PAPs, are required in the activation mixture to generate active PO.

Although it has been shown that all the insect PPAEs cut proPO at Arg^{51}, the need for a cofactor to generate active PO is inconsistent. *M. sexta* PAPs and *H. diomphalia* PPAF-I require SPHs for generating active PO, whereas *B. mori* PPAE does not.

Serine proteinase homologs (SPHs)

Some SPHs are similar to PPAEs in that they contain a clip domain at amino terminus and SP-like domain at carboxyl terminus. However, they lack proteolytic activity because the active site Ser is replaced by Gly. SPHs have been isolated from hemolymph of *Manduca sexta* (Yu et al., 2003) and hemocyte lysate of *Holotrichia diomphalia* (PPAF-II) (Kwon et al., 2000), and they may act as a cofactor of PAPs or PPAF-II to generate active PO in these two insects. However, their mode of action is not understood. Neither is it known whether *M. sexta* SPH-1 or SPH-2 alone is active as an auxiliary factor – the purified SPHs exist as oligomers with an average molecular mass of 790 kDa (Wang and Jiang, 2004b). SPHs may interact either with PAPs, proPO, or both, and such binding may lead to a favorable conformation for exhibiting PO activity. Since *M. sexta* SPHs and IML-2 co-purify on an affinity column, their interaction may localize, through IML-2 (an LPS-binding lectin), proPO activation near Gram-negative bacteria (Yu et al., 2003). SPHs are synthesized as precursors and require activation by limited proteolysis. For instance, *H. diomphalia* PPAF-II is activated by PPAF-III, a clip-domain SP (Kim et al., 2002).

Serpins

Serpins are typically 45-50 kDa serine proteinase inhibitors found in plants, invertebrates, and vertebrates (Silverman et al., 2001). In mammals, they regulate blood coagulation, fibrinolysis, complement activation, and inflammatory responses. Serpins have also been isolated from the hemolymph of arthropods (Kanost, 1999). They also regulate defense responses, such as hemolymph coagulation. Serpins negatively regulate

the proPO activation pathway by inhibiting its SP components. The acyl-enzyme complex formed between serpin and SP is stable at 95°C in SDS-sample buffer (Church et al., 1997; Kanost, 1999).

Tertiary structure of serpins reveals a reactive site loop near the carboxyl end. The amino acid residue before the scissile bond, also known as the P_1 residue, is important in binding to the primary specificity pocket of its target SP. Studies have shown that mutation in the reactive site loop, especially the P1 residue, significantly affects the inhibitory selectivity of a serpin. Serpins have been identified in *B. mori*, *M. sexta*, *L. migratoria*, *A. gambiae,* and *D. melanogaster* (Sasaki, 1991; Takagi et al., 1990; Narumi et al., 1993; Kanost et al., 1989; Kanost and Jiang, 1997; Gan et al., 2001).

In *M. sexta*, six serpins have been found. In serpin-1, 12 variants are generated from a single gene through usage of alternative exon 9's (Kanost et al., 1989; Jiang et al., 1994; Kanost et al., 1995; Jiang et al., 1996). Only serpin-1J blocks proPO activation by inhibiting all three PAPs (Jiang et al., 2003b). Recently, we discovered that serpin-3 also inhibits all the PAPs and serpin-6 specifically inhibits PAP-3 (Zhu et al., 2003; Wang et al., 2004). *B. mori* antitrypsin inhibits a hemolymph SP but such inhibition has no effect on proPO activation (Ashida and Sasaki, 1994).

Prophenoloxidase (proPO)

PO (monophenol, *L*-dopa: oxygen oxidoreductase, EC 1.14.18.1), a copper-containing tyrosinase, plays important roles in melanization, wound healing, and cuticle sclerotization. Tyrosinase is ubiquitously present in all forms of life from prokaryotes to eukaryotes (Mason, 1965). PO is synthesized as an inactive zymogen, named proPO.

The conversion from proPO to PO is mediated by a cascade of SPs in insects, most of which have not been identified yet. These SPs are sequentially activated by limited proteolysis when PAMPs are recognized by PRRs (Ashida and Yamazaki, 1990; Ashida and Brey, 1998). To minimize the cytotoxicity of quinones and other reactive intermediates to host tissues and cells, proPO activation and PO activity have to be regulated as a local, transient, reaction against nonself.

Insect proPO is homologous to hemocyanins of horseshoe crab, spiders, and crustaceans (Van Holde et al., 1995). While oxygen transport is carried out by hemocyanins in some arthropods, this function is carried out via diffusion by the tracheal system in insects. In *B. mori* and *M. sexta*, proPO produced by oenocytoids is present in the plasma. Since proPO does not contain a signal peptide for secretion, its release mechanism remains to be investigated (Iwama and Ashida, 1986). Purified *M. sexta* proPO contains two polypeptides in equal amounts. cDNA cloning indicated that proPO-p1 (78 kDa) and -p2 (80 kDa) are 50% identical to each other whereas they are 78% identical to *B. mori* proPO-p1 and –p2, respectively. Size exclusion high-pressure liquid chromatography (HPLC) analysis shows that *M. sexta* proPO exists as monomeric, dimeric, trimeric or multimeric structures depending on the ionic strength (Jiang et al., 1997b). Non-denaturing gel electrophoretic analysis has revealed two forms of proPO with different mobility. ProPO cDNAs have also been isolated from many other insects, including *D. melanogaster* (Fujimoto et al., 1995), *A. gambiae* (Jiang et al., 1997a), and *H. diomphalia* (Kwon et al., 1997).

Activation of proPO

Under the physiological conditions, proPO activation is catalyzed by PPAE through limited proteolysis. However, proPO can also be activated by incubation with detergents or alcohols (Asada et al., 1993; Hall et al., 1995). The latter process, not involving cleavage of any peptide bond, appears to be caused by a conformational change in proPO. As the physiological relevance of such SP-independent proPO activation is unclear, we will only discuss the mechanisms of SP-mediated proPO activation in different insects.

By cleavage at Arg^{51}, *B. mori* PPAE itself activates proPO and yield active PO (Satoh et al., 1999). On the other hand, PAPs/PPAF-I requires clip-domain SPHs to produce active PO in *M. sexta* and in *H. diomphalia*. *H. diomphalia* PPAF-I alone cuts proPO-p1 and -p2 at Arg^{51} to form an inactive intermediate. Active PO (60 kDa) is generated only after proPO-p1 is cleaved at Arg^{165} by PPAF-I in the presence of PPAF-II, a clip-domain SPH (Kwon et al., 2000; Lee et al., 1998a). Sequence alignment indicates that the Arg^{165} equivalent is present in *M. sexta* proPO-p1 but not in *B. mori* proPO-p1. An easter-like SP, *H. diomphalia* PPAF-III, cleaves the precursor of PPAF-II to form an active cofactor (Kim et al., 2002). In *M. sexta,* PAPs alone cleave proPO at Arg^{51} and generate a little PO activity (Jiang et al., 1998; Jiang et al., 2003a; Jiang et al, 2003b). In the presence of SPH-1 and SPH-2, cleavage at Arg^{51} was somewhat enhanced while there was a major increase in PO activity (Yu et al., 2003). The secondary cleavage in proPO-p1 was also observed sometimes (Wang et al., unpublished result), but its role in generating active PO is not clear. These results suggest that there could be different mechanisms for proPO activation in different insects.

Antimicrobial peptides and their transcriptional activation

Different types of antibacterial peptides are found in insects at different stages of their life cycles. These include lysozymes, attacins, cecropins, and defensins (Bulet et al., 1999).

Lysozyme is a 14 kDa enzyme that hydrolyzes peptidoglycan of Gram-positive bacteria. It may also degrade the bacterial component left after phagocytosis and nodulation (Bulet et al., 1999).

Cecropins are small 3 kDa, amphipathic proteins. These are released into the hemolymph in response to Gram-positive and Gram-negative bacteria. They penetrate the plasma membrane of the pathogens, perturb the electrochemical ion gradient, and kill the bacteria. Cecropins are found only in lepidopteran and dipteran insects.

Insect defensins are similar to defensins from human neutrophil and macrophages, which affect Gram-positive bacteria only. Attacins (20 kDa) interfere with the transcription of a membrane protein found in outer surface of Gram-negative bacteria.

Hemolin (48 kDa), a member of the immunoglobulin superfamily, is discovered in *Hylophora cecropia* and *M. sexta*. It is composed of 4 immunoglobulin domains and does not kill bacteria directly. Hemolin may act as an opsonin by binding to LPS and hemocytes (Yu, et al., 2002a).

In *D. melanogaster* adults, synthesis of antimicrobial peptides and other immune proteins is regulated by Toll and Imd pathways. The Toll pathway is activated in response to fungal or Gram-positive bacterial infection, whereas the Imd pathway is responsive to Gram-negative bacterial infection. Toll, a transmembrane receptor, is involved in the dorsoventral patterning of early embryo as well as immune responses in

adults. Toll pathway in hemolymph is triggered by an extracellular SP cascade which activates spätzle. Interaction of spätzle with Toll initiates an intracellular signaling cascade that eventually causes degradation of Cactus and nuclear translocation of DIF (Hoffmann and Reichhart, 2002). DIF is a member of the Rel family transcription factors that bind to NF-κB motif and activate transcription of immune proteins, such as drosomycin. The Imd pathway can be activated by Gram-negative bacteria through PGRP-LC, a cell surface PRR. Another Rel family member, Relish, is activated by a proteolytic cleavage and translocated into the nucleus where it turns on the transcription of another set of immune genes. These two pathways share many similarities with TLF (Toll-like receptor factor) and TNF (tumor necrosis factor) pathways in mammals, which cause activation of transcription factor NFκB (Hoffmann and Reichhart, 2002).

CHAPTER III

MATERIALS AND METHODS

Purification of M. sexta proPO, SPHs, serpin-1J

As reported previously, *M. sexta* proPO was isolated from the larval hemolymph (Jiang et al., 1997b). SPHs consisting of SPH-1 and SPH-2 were co-purified from hemolymph of the prepupae according to Wang and Jiang (2004 b). *M. sexta* serpin-1J was expressed and purified as a recombinant soluble protein from *Escherichia coli* (Jiang and Kanost, 1997c). Recombinant serpin-1J was further purified by ammonium sulfate (AS) fractionation and anion exchange chromatography on Biologic Dual-Flow Protein Purification System (Jiang et al., 2003b).

Detection of PAP-1 by an amidase activity assay and immunoblot analysis

To determine the PAP-1 catalytic activity acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR*p*NA) was used as a chromogenic substrate. Amidase activity assay was performed by using 150 µl of 25 µM of IEAR*p*NA in 0.1M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, pH 8.0 to each well. Absorbance at 405 nm was measured for 20 min continuously on a microtiter plate reader. Since the cuticle extract may contain other proteinases that may hydrolyze the same substrate. To detect PAP-1, SDS-PAGE and immunoblot analyses were performed by using 1: 2000 dilution of antibody of PAP-1.

PO activity assay

PO activity assay was performed by adding 150 µl of 2 mM of dopamine in 50 mM sodium phosphate at pH 6.5 to each well. Absorbance at 470nm was measured for 20 min continuously on the microtiter plate reader.

Cuticle collection and extraction

The integuments of *M. sexta* prepupae with metathoracic brown bars were separated from hemolymph, gut, and fat body by dissection (Jiang et al., 2003a). The integuments were washed twice in chilled extraction buffer (0.1 M Tris-HCl, 0.5 M NaCl, 0.002% Phenylthiourea (PTU), 1 mM benzamidine, pH 7.5) and stored at -80°C. Proteins were extracted from the homogenized cuticles and fractionated with 15-40% saturated AS (Jiang et al., 2004). Fractionated proteins were dissolved in HT buffer (pH 6.8, 20 mM potassium phosphate, 0.5 M NaCl) and stored at -80°C.

Optimized purification of M. sexta PAP-1

All procedures for purification of PAP-1 were carried out at 4°C to prevent the loss of enzymatic activity. The 15-40% AS fraction of cuticular proteins was further activated by *Micrococcus luteus* (0.5 mg/ml) and curdlan (0.5 mg/ml) for 10 min in the presence of 0.001% PTU. After removal of the flocculent materials by centrifugation at 40,000×*g* for 15 min, the recovered supernatant was replenished with 0.5 mM *p*-aminobenzamidine and 0.001% Tween-20, and dialyzed against HT buffer (20 mM potassium phosphate, pH 6.8, 0.5 M NaCl) supplemented with 0.001% PTU, 0.5 mM

benzamidine, and 0.001% Tween-20 (1.0 liter for 8 h, twice).

After centrifugation at 40,000×*g* for 30 min, the protein sample was applied to a hydroxylapatite column (2.5 cm i.d. x 7 cm, Bio-Rad), equilibrated in HT buffer. Following a washing step with 100 ml HT buffer, bound proteins were eluted at a flow rate of 0.4 ml/min for 5 h with a linear gradient of 20-150 mM potassium phosphate (pH 6.8) in 0.5 M NaCl.

Combined PAP-1 fractions (40 ml) were mixed with 8 volumes of DS buffer (0.001% Tween-20, 10 mM potassium phosphate, pH 6.4) containing 0.001% PTU and 0.5 mM benzamidine. The diluted sample (360 ml) was applied to a dextran sulfate-Sepharose CL-6B column (1.5 cm i.d. x 20 cm, Sigma) 0.5 ml/min for 12 h. After washing the column with 90 ml buffer, bound proteins were eluted at a flow rate of 0.45 ml/min for 4.4 h with a linear gradient of 0-1.0 M NaCl in DS buffer. The pooled PAP-1 fractions (35 ml) were concentrated on a Centricon (Amicon) and precipitated with 50% AS.

After centrifugation at 40,000×*g* for 30 min, the precipitated proteins were dissolved in 4.0 ml of S100 buffer (0.001% Tween-20, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4) containing 0.5 mM *p*-aminobenzamidine, and then applied to a Sephacryl S100-HR column (2.5 cm i.d x 100 cm, Amersham Biosciences). The column was eluted with S100 buffer at a flow rate of 0.7 ml/min, and fractions were collected at 2.8 ml/tube. The fractions were pooled, replenished with 0.5 mM of *p*-aminobenzamidine, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and loaded to a Jacalin-agarose column (5.0 ml, Amersham Biosciences). Following a washing step with J buffer (0.001% Tween-20, 1 mM $CaCl_2$, 1 mM of $MgCl_2$, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4), the fractions were eluted in J

buffer containing 0.2 M melibiose (Sigma) at 0.5 ml/min and collected 1.5ml per tube.

Characterization of PAP-1

To determine the purity and molecular weight of PAP-1 we performed SDS PAGE analysis followed by silver staining. Purified PAP-1 (12 µl) was mixed with 3 µl of 5x SDS sample buffer with and without dithiothreitol and boiled for 5 min. The sample was loaded and separated by 12% gel. For immunoblot analysis (15% gel) membrane was developed by using 1: 2000 diluted antiserum against PAP-1 as the first antibody. MALDI-TOF mass spectrometry of PAP-1 was carried out as previously described (Jiang et al., 2003a)

To determine the optimal pH of PAP-1 based on amidase activity, PAP-1(1.0 µl, 20 ng/µl) was incubated with 150 µl, 25 µM IEAR*p*NA in 1:5 diluted PBE96 (Polybuffer 96, Amersham Biosciences) adjusted to various pH's. Amidase activity was measured by using IEAR*p*NA as a substrate (Jiang et al., 2002a). To determine the heat stability of PAP-1, PAP-1 (1.0µl,20ng/µl) was placed at different temperatures (0, 10, 20, 30, 40, 50, 60, and 70°C) for 5 min and the amidase activity was measured by using 150 µl, 25 µM IEAR*p*NA in 0.1 M NaCl, 0.1 M Tris-HCl, pH 7.8. The residual amidase activity was plotted against the preincubation temperatures for determining the heat stability of PAP-1.

Effect of SPHs on proPO activation

To a fixed amount of PAP-1 (20 ng) and proPO (100 ng), varying amounts of SPHs (0.52, 1.04, 2.08, 4.16, 8.33, 16.67, 33, 66 ng) were added. The final volume was

adjusted to 30 µl with buffer. The reaction mixture was split into three wells containing 10 µl each and incubated on ice for 60 min and PO activities were measured and plotted on the Y-axis. Amount of SPHs was plotted on the X-axis. Nonlinear regression analysis of the experimental data was performed by using Prism 3.0 (GraphPad Software, Inc.).

Effect of PAP-1 on proPO activation was determined by following the same procedure as described above. To the 20 ng of SPHs and 100 ng of proPO, varying amounts of PAP-1 (0.52, 1.04, 2.08, 4.16, 8.33, 16.67, 33, 66 ng) were added. PO activity and data analysis were carried out similarly.

Examination of optimal conditions for proPO activation

To determine the optimal pH for proPO activation, we mixed PAP-1 (20 ng), SPHs (20 ng) and proPO (0.1 µg/µl) and diluted in 1: 5 PBE96 adjusted to various pHs and placed on ice for 60 min. PO activity was measured using dopamine as substrate.

Heat stability of PAP-1 and SPHs was determined by preincubating PAP-1(20 ng) and SPHs (20 ng) at different temperatures (0, 10, 20, 30, 40, 50, 60, and 70°C) for 5 minutes. ProPO (0.1µg/10µl) was added on ice for 1 h prior to PO activity determination.

To examine the time course of proPO activation at different temperatures, we mixed PAP-1 (20 ng), SPHs (20 ng) and proPO (0.1µg/10µl) and adjusted the volume to 60 µl with the buffer. Reaction mixture was split into three wells containing 20 µl each and placed at different temperatures (0, 10, 20, and 30 °C) for different periods of time. PO activity was assayed and plotted against reaction time.

To study the effect of ionic strength on proPO activation, we mixed PAP-1 (20 ng), SPHs (20 ng) and proPO (0.1 μg/μl) and adjusted the volume to 60 μl with the buffer containing different concentrations (0, 50, 100, 150, 200, and 300 mM) of NaCl. Reaction mixture was equally split into three wells and incubated on ice for 1 h prior to PO activity measurement. To determine the effect on cleavage of proPO we mixed the activation mixtures in 0.001%of PTU and subjected to SDS-PAGE.

To test the effect of ionic strength on PO activity, we mixed PAP-1 (20 ng), SPHs (20 ng) and proPO (0.1 μg/μl) and adjusted the volume to 60 μl with the buffer and equally split the reaction into three wells and incubated on ice for 1 h and added dopamine substrate solutions containing increased concentrations (0, 50, 100, 150, 200, 250 mM) of NaCl for PO activity determination.

Measurement of enzymatic properties of PAP-1

To determine the V_{max} and Michaelis constant (K_M) of PAP-1 toward IEAR*p*NA we incubated purified PAP-1 (2.0 μl, 20 ng/μl) and buffer (8.0 μl, 20 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.5) with the SPHs (1.0 μl, 20 ng/μl) or H_2O (1.0 μl) in microplate wells for 5 min on ice. The initial velocity of the reaction was measured using 150 μl IEAR*p*NA (25 - 400 μM) in the same buffer (Jiang et al., 2003a). For determination of K_M, linear regression was performed from the Lineweaver-Burk plot by the least-squares method. Similarly K_M of the PAP-1 for proPO was measured in the presence of its cofactor: PAP-1 (1.0 μl, 20 ng/μl), SPHs (1.0 μl, 20 ng/μl), and the buffer (8.0 μl) were incubated with various amounts of proPO (12.5-400 ng) in 10 μl, 20 mM Tris-HCl, pH

8.0 for 20 min on ice. PO activities in 20 µl reaction mixtures were measured and plotted similarly.

Inspection of the relationship between proPO cleavage and PO activity

ProPO (0.1 µg/10µl) was incubated with buffer, PAP-1 (40 ng/µl), or a mixture of PAP-1 and SPHs (40 ng + 40 ng) at room temperature for 15 and 30 min (see Fig. 9 for details). Extent of cleavage of proPO was analyzed by SDS-PAGE. For SDS PAGE analysis, the samples were mixed with 0.001%PTU, treated with sample buffer and separated on 10% gel and visualized by silver staining. In the duplicate reactions (without PTU), PO activities were determined and compared with the relative amounts of cleaved proPO.

Examination of the significance of limited proteolysis in proPO activation

To study the importance of cleavage of proPO, 100 ng proPO and 40 ng SPHs were incubated with buffer, APMSF-inactivated PAP-1 (40 ng), or active PAP-1 at 0°C for 1 h (see Fig. 10 for details). The reaction mixtures were analyzed by SDS-PAGE followed by immunoblot using proPO antibodies. The extent of proPO cleavage was examined and compared with corresponding PO activities in the duplicate reactions.

Investigation of a possible interaction between cleaved proPO and SPHs in the presence of inactivated PAP-1

We incubated PAP-1 (20 ng) and proPO (100 ng) on ice. After 20 min, aliquots of the reaction mixtures were taken for PO activity assay, IEARase determination, and

immunoblot analysis using proPO antibodies. Recombinant serpin-1J (3 μg) was added to inhibit the PAP-1 activity for 15 min. In the duplicate reactions, buffer was added as a control. Samples were taken from the reaction mixtures at 5 and 15 min for activity assays and immunoblot analysis. After SPHs (20 ng) were added, the reaction mixtures were further incubated for 15 min at 0°C and subjected to the activity and gel analysis. The same experiment was repeated but instead of using serpin-1J to inactivate PAP-1, APMSF (8 mM) was added. Immunoblot analysis was performed by using 1: 2000 diluted antiserum against proPO as the first antibody.

Possible interaction of SPHs with cleaved proPO in the presence of active PAP-1

To test whether SPHs can interact with cleaved proPO to generate PO activity in the presence of active PAP-1, we mixed and incubated PAP-1 (40 ng) and proPO (100 ng) for 90 min to get more than 80% of hydrolysis of proPO. At 90 min, SPHs (40 ng) were added and incubated for 10 more min. In duplicate reactions as a control we mixed proPO, PAP-1 and SPHs at the same time and incubated for 100 min on ice. At 90 and 100 min the reaction mixtures were analyzed by SDS-PAGE and PO activity assays.

Effect of M. sexta SPHs on silkworm proPO activation

Silkworm proPO (0.2 μg, 20μl) was incubated with buffer, PAP-1 (40 ng, 4μl), or PAP-1 and SPHs (40 ng, 2μl). The final volume was adjusted to 26 μl by adding buffer (20 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$) and incubated at room temperature for 15 min. Extent of cleavage of proPO was analyzed by SDS-PAGE. For SDS PAGE analysis, the samples were mixed with 0.001% PTU, treated with sample buffer and separated by

SDS-PAGE, and visualized by silver staining. 15 µl of each sample was loaded and separated on 7.5% gel. For immunoblot analysis, 1: 2000 diluted antiserum against *M. sexta* proPO was used as the first antibody.

SDS-PAGE and immunoblot analyses

Laemmli method was used to separate the proteins and followed by silver staining. For immunoblot analysis after SDS–PAGE, proteins were transferred to nitrocellulose membrane and blocked with 5% skim milk in TBS for 15 min. The membrane was incubated in rabbit antiserum against PAP-1 or proPO -p1, and -p2 (1: 2000 dilutions in 1% milk in TBS) for 2 h. After washing the membrane in 1X TBS for three times for 10 min the secondary antibody (1: 2000 diluted goat anti-rabbit IgG (Bio-Rad) was added and incubated for 2 h. Membrane was washed again three times in 1X TBS for 10 min and developed by a color reaction catalyzed by alkaline phosphatase.

CHAPTER IV

RESULTS

Purification of PAP-1

We previously reported partial purification of *M. sexta* PAP-1 from the prepupal cuticles (Jiang et al., 1998). Active PAP-1 was subsequently isolated from a baculovirus expression system for a functional analysis of *M. sexta* SPH-1 and SPH-2 (Yu et al., 2003). However, the recombinant enzyme was insufficient for studying interactions among proPO, PAP-1, and SPHs. In this work, we attempted to improve the purification scheme and isolate PAP-1 from the cuticles of *M. sexta* prepupae. Immunoblot analysis using PAP-1 antibodies and amidase assay using IEAR*p*NA allowed us to detect PAP-1 in the column fractions.

After dialysis, 15-45% ammonium sulfate fraction of the cuticular extract was separated into two protein peaks by hydroxyapatite chromatography: one in the flow through and the other in the eluted fractions (Fig. 1A). The second A_{280} peak coincided with the amidase activity peak in fractions 29-41, which corresponded to 60-115 mM potassium phosphate. Immunoblot analysis indicated that PAP-1 correlated with the amidase activity (data not shown). PAP-1 fractions were pooled and loaded onto the dextran sulfate (DS) Sepharose column. PAP-1, present in the DS-bound fractions 17-25, was separated from other proteins including SPHs (Fig. 1B).

The active fractions were pooled, concentrated and resolved on the gel filtration column. The amidase activity (Fig. 1C) and immunoreactivity (data not shown) were detected in fractions 36-56. The active fractions 44-53 were enriched and purified by affinity chromatography on the Jacalin-agarose column (Fig. 1D). Jacalin-bound fractions 8-12 contained PAP-1 with a high activity.

Characterization of PAP-1

SDS-PAGE analysis of the purified PAP-1 indicated that the proteinase migrated as two bands with apparent molecular masses of 17 and 31 kDa under reducing condition (Fig. 2A, lane 1). PAP-1 migrated as a single band at 44 kDa under non-reducing condition (Fig. 2A, lane 3), indicating that the two polypeptides were covalently attached by a disulfide bond. cDNA cloning also suggested that PAP-1 is composed of a clip domain (13 kDa) at the amino terminus and a catalytic domain (31 kDa) at the carboxyl terminus (Jiang et al., 1998). The smaller polypeptide corresponds to residues 1-108 of the mature protein. It has a calculated mass of 11,560 Da, less than the apparent mass (17 kDa) on the SDS-polyacrylamide gel. This abnormal behavior is mainly due to the clip domain structure (Jiang et al., unpublished data). The light chain did not stain well (Fig. 2A), but was strongly recognized by PAP-1 polyclonal antibodies (Fig. 2B, lane 1). In contrast, PAP-1 polyclonal antibodies did not react well with the catalytic domain (31 kDa) (Fig. 2B, lane 1) and the 44 kDa unreduced form (Fig. 2B, lane 2).

The molecular mass of PAP-1 was determined by MALDI-TOF mass spectrometry to be 39,810 +/- 20 Da (Fig. 3), slightly higher than the calculated value (39,550 Da). The difference is probably due to glycosylation of PAP-1 at specific Thr

residues. This is supported by the fact that PAP-1 binds to Jacalin. Since no other major mass peaks were identified on the spectrum, we suggested that the PAP-1 preparation contained mostly PAP-1.

The optimal pH and temperature for PAP-1 activity were examined using IEAR*p*NA. The amidase activity was the highest at pH 8.0 (Fig. 4A). At a physiological pH (6.5-7.0) of the larval hemolymph, this activity reduced to about 60% of the maximum. PAP-1 showed good thermal stability below 50ºC. It was stable up to 45ºC, and 20% and 100% of the activity was lost at 50 and 60ºC, respectively (Fig. 4B). The isoelectric point of PAP-1 was around 5.6, slightly lower than the calculated one (5.9).

Effect of SPHs and PAP-1 on proPO activation

When increased amounts of SPHs were added to the fixed amount of PAP-1 and excess proPO, we detected higher levels of PO activity (Fig. 5A). Similarly, PO activities increased (Fig. 5B), when increased amounts of PAP-1 were added to excess proPO and fixed SPHs. From non-linear regression analysis, we found that the molecular associations fit the two-site binding model with an r^2 of 0.99 and 0.93, respectively. The optimal molar ratio of PAP-1 and SPHs for proPO activation was 1: 1.5, and further increase in PAP-1 did not yield more PO activity.

Optimization of conditions for proPO activation

The optimal pH for proPO activation by PAP-1 and SPHs was around 8.0. PO activity increased from pH 6.0 to 8.0 and was highest at pH 8.0. At pH 6.0 and 9.0, 30% and 45% of the maximum PO activity was detected (Fig. 7A). At a physiological pH of

the larval hemolymph, 80% of the maximum PO activity was maintained. These results are consistent with the reduction in the PAP-1 amidase activity (Fig. 4A).

In order to study the thermal stability of PAP-1 and SPHs, we mixed and pre-incubated them at different temperatures (0-70°C) for 5 min. The mixtures were further incubated with proPO at 0°C for 1 hr. As the preincubation temperature increased, PO activity decreased steadily. At 40°C, 80% of the maximum PO activity was observed, whereas at 60°C the activity was completely lost (Fig. 7B). The mixture of PAP-1 and SPHs was stable below 40°C.

As reported earlier (Wang and Jiang, 2004b), when PAP-3, SPHs, and proPO were incubated at different temperatures for 1 hr, higher PO activities were observed at 0°C and 10°C, rather than 20°C or 30°C. PO activities were 60% and 20% of the maximum, respectively. Since PAP-1, PAP-3 and SPHs have good thermal stability up to 30°C; we further studied the time course of proPO activation at 0, 10, 20, and 30°C. We found that at 0°C, the PO activity increased steadily with time and reached the highest level (8.8 U) in 50 min (Fig. 7C). At 10, 20, and 30°C, PO activity developed faster in the beginning and reached their peaks (12.6, 16.7, and 10.8 U) in 35, 15, and 10 min, respectively. At higher temperatures (20°C and 30°C), PO activity loss was more rapid than at lower temperatures (0°C and 10°C). This result suggested that PO activity is more stable at lower temperatures.

High ionic strength in the proPO activation mixtures reduced final PO activity level (Wang and Jiang, 2004). In this work, we determined whether ionic strength affects proPO activation or PO activity (Fig. 7D). For proPO activation, we incubated PAP-1, SPHs, and proPO (i.e. proPO activation mixtures) for 1 h in the presence of increasing

concentrations of NaCl and analyzed the PO activity. For PO activity, we incubated PAP-1, SPHs and proPO for 1 h. After that, we measured PO activity using dopamine substrate solutions containing different levels of NaCl. In both the cases, PO activity was lower at higher salt concentrations and 50% of the maximum PO activity occurred at 100 mM of NaCl (Fig. 7D). These results confirmed that ionic strength affects PO activity but its effect on proPO activation was still uncertain. Therefore, we examined the effect of ionic strength on proPO cleavage (Fig. 8). Immunoblot analysis showed that in the presence of 300 and 400 mM NaCl, proPO cleavage was reduced to nearly 50%.

Effect of SPHs on the V_{max} and K_m of PAP-1 towards a synthetic substrate

To determine whether SPHs can bring any change in the amidase activity of PAP-1, we incubated PAP-1 in the presence or absence of SPHs and measured the amidase activity by using IEAR*p*NA as a substrate. We observed no change in the level of amidase activity of PAP-1 (Fig. 6A). SPHs had no effect on the catalytic activity of PAP-1 towards the synthetic substrate.

We further examined the effect of SPHs on the Michaelis constant (K_M) of PAP-1 towards IEAR*p*NA. We incubated PAP-1 in the presence or absence of SPHs and measured the amidase activity at various concentrations of IEAR*p*NA. In both the cases, the activity data fit the Michaelis-Menten equation with an r^2 of 0.98 and 0.99, respectively. The presence of SPHs did not bring any significant change in K_M. The K_M's with and without SPHs were 201 ± 18 and 248 ± 39 μM respectively (Fig. 6B), which are not significantly different.

Determination of K_M of PAP-1 towards proPO

To determine the K_M of PAP-1 toward its natural substrate, we incubated PAP-1 and SPHs with varying amounts of proPO and measured PO activity. The activity data fit the Michaelis-Menten equation with an r^2 of 0.99. The apparent K_M for proPO was 16.5 ± 6.0 μg/ml, close to its physiological concentration in larval hemolymph (Fig. 6C). Since PAP-1 alone produced little active PO, we could not compare that with the K_M of PAP-1 towards proPO in the absence of SPHs.

Is there a direct relationship between proPO proteolysis and activation?

Yu et al. (2003) previously reported that recombinant PAP-1 alone did not efficiently cleave proPO. But, in the presence of SPHs, cleavage of proPO was more complete (~30%) and accompanied with a high level of PO activity (22 U). Here, we repeated the same experiment using the purified cuticular PAP-1 to test whether cleavage of proPO directly correlated with PO activity (Fig. 9). We incubated proPO, proPO with PAP-1, proPO with PAP-1 and SPHs at room temperature for 15 and 30 min. On SDS-PAGE, proPO -p1, and -p2 and cleaved proPO migrated slightly faster than expected from their masses. In the absence of SPHs, PAP-1 cleaved more than 30% of proPO and generated lowered band (74 kDa) at 15 min, but did not produce significant PO activity (1.7 U). In the presence of SPHs, a 25-fold increase in PO activity (44 U) was observed, along with a moderate increase in proPO cleavage. At 30 min, PAP-1 alone cleaved more than 70% of proPO (Fig. 9A, lanes 5) and yielded low PO activity (2.4 U). In the presence of SPHs, higher levels of PO activity (23 U) with increase cleavage products (1.5 fold) were also observed. There was no proPO cleavage and PO activity at 15 and

30 min in the control of proPO only. These results indicated that cleavage of proPO did not directly correlate with PO activity and that SPHs are required along with PAP-1 to convert proPO to active PO.

Is cleavage of proPO important for its activation?

While proPO cleavage and PO activity did not have a direct correlation, we also wanted to test whether or not the proteolysis is essential for proPO activation by PAP-1 and SPHs. It is known that certain organic chemicals (*e.g.* detergents and alcohols) or polypeptides (*e.g*. antimicrobial peptides, serine proteinase precursors) activate proPO by inducing a conformational change without proteolysis (Asada et al, 1993; Hall et al, 1995). Is it possible that PAP-1 and SPHs activate proPO in the same way? To test this possibility, we first inactivated PAP-1 with APMSF, an irreversible serine proteinase inhibitor. Later, after SPHs and proPO were incubated with the inactivated PAP-1 on ice for 1 h, proPO was not cleaved and little PO activity (0.5U) was generated (in the presence of APMSF-inactivated PAP-1 and SPHs) (Fig. 10). In contrast, in the presence of active PAP-1 and SPHs, more than 50% of cleavage of proPO and a high level of PO activity (44 U) were observed. This result clearly showed that cleavage of proPO by PAP-1 is critical for generating PO activity.

Formation of PO oligomers in the presence of SPHs:

The above results suggest that PO generated in the absence or presence of SPHs by PAP-1 must be different somehow. They may exist in different association states. Therefore, to understand the active PO, we incubated proPO (100 ng) and PAP-1, in the

presence or absence of SPHs (40 ng) on ice for 1 h. The reaction mixtures were separated by electrophoresis on a 6% SDS-polyacrylamide gel followed by immunoblot analysis (Fig. 11A). In the duplicate reactions, PO activity was determined using dopamine (Fig. 11B). After proPO had been incubated with PAP-1 for 1 h, cleaved proPO (74 kDa) was generated with a low level of PO activity (1.7 U) (Fig. 11B). In the presence of the SPHs, the cleavage of proPO increased 3 fold (Fig. 11A, lane 3) with high level of PO activity (67 U) (Fig. 11B). In addition, proPO antibodies also recognized three high molecular weight bands: one at 140 kDa and two at greater than 250 kDa (Fig. 11A, lane 3). We did not detect any high molecular weight bands in the control of proPO only, or in SPHs with proPO. A similar observation was reported in PO activated by PAP-2 and SPHs (Jiang et al., 2003a). This indicated that these are covalent protein complexes that contain PO. Thus, it supports the hypothesis that active PO may be in a different association state and form high molecular weight species.

Are SPHs and PAP-1 required at the same time for proPO activation?

Jiang et al. (1998) proposed that proPO activation by PAP-1 requires a protein cofactor, which was found to be SPH-1 and SPH-2 later (Yu et al., 2003). However, no evidence was provided that SPHs and PAP had to be present at the same time – a prerequisite for SPHs to serve as "PAP cofactor". While increased cleavage of proPO certainly suggest an interaction of SPHs with proPO and/or PAP-1 (Wang and Jiang, 2004b), we wanted to examine whether proteolysis of proPO by PAP-1 and binding of cleaved proPO by SPHs are two independent steps in proPO activation. In other words,

can SPHs themselves bind to cleaved proPO and generate active PO, and not act as a "cofactor" to PAP-1?

To test this concept of cofactor, we designed an experiment to separate PAP-1 from SPHs by an irreversible serine proteinase inhibitor, serpin-1J. We pre-incubated PAP-1 and proPO on ice for some time till sufficient proPO was cleaved, inactivated PAP-1 by adding serpin-1J, incubated the reaction mixture with SPHs, and then determined PO activity. After 20 min incubation of proPO with PAP-1, we observed 20% cleavage of proPO (Fig. 12B, lane 2) and little PO activity (2.2 U) (Fig. 12A). At 5 and 15 min after serpin-1J was added, PAP-1 amidase activity was completely blocked (0.0 U), and there was no significant change in PO activity (1.5 U). As a control, a buffer was added to the other half of the reaction mixtures (containing active PAP-1) and we did not observe much change in PAP-1 amidase and PO activities (~2.0 U, 2.0 U). We then added same amount of SPHs to both reaction mixtures (control and serpin-1J) and incubated further for 15 min. In serpin-1J-treated sample (containing inactive PAP-1), we detected little PO (1.7 U) and amidase activities (0.04 U). In the control reaction (containing active PAP-1), significant PO and amidase activities were observed (17.3 U, 2.1 U).

As a serpin forms high M_r covalent complex with its cognate proteinase, there is a possibility that SPHs were unable to interact with cleaved proPO due to the complex interference. Therefore, we repeated the same experiment by replacing serpin-1J with APMSF, a small M_r inhibitor not expected to interfere with the protein-protein interactions. We obtained a similar result, APMSF completely blocked the amidase activity of PAP-1 (0.04 U). After SPHs were added, they failed to generate PO activity

(1.62 U), whereas in the control (containing active PAP-1) a high level of PO activity was observed (13.7 U) (Fig. 13A). These results indicated that SPHs cannot generate PO activity by binding to cleaved proPO, even though APMSF-inactivated PAP-1 was also present. SPHs and active PAP-1 are required at the same time to activate proPO.

Can SPHs interact with cleaved proPO in the presence of active PAP-1 to generate PO activity?

While SPHs did not generate active PO by interacting with cleaved proPO and inactivated PAP-1 is it possible that interaction of SPHs with cleaved proPO requires active PAP-1? To test this possibility, we used PAP-1 to hydrolyze proPO to >80%, then added SPHs, incubated for another 10 min, measured PO activity, and performed SDS-PAGE analysis to examine the proPO cleavage. After 90 min of incubation on ice, PAP-1 cleaved >80% of proPO (Fig. 14A, lane 2) and yielded a low level of PO activity (1.5 U) (Fig. 14B). Further incubation with SPHs for 10 min brought a small increase in cleavage (Fig. 14A, lane 3) and PO activity (2.6 U) (Fig. 14B). In the control, where we mixed proPO, PAP-1, and SPHs at the same time and incubated for 100 min, we observed a similar proPO cleavage (Fig. 14A, lane 4) but a much higher level of PO activity (10.0 U)(Fig. 14B). Therefore, the SPHs did not interact with cleaved proPO to generate PO activity even in the presence of active PAP-1.

Can M. sexta SPHs and PAP-1 activate silkworm proPO?

ProPO activation in *M. sexta* differs from that in *B. mori*: the silkworm proPO-activating enzyme (PPAE) does not require other proteins (Satoh et al., 1999). To test

whether *M. sexta* SPHs could enhance silkworm proPO activation, we incubated *B. mori* proPO and *M. sexta* PAP-1 at room temperature for 15 min. In the absence of *M. sexta* SPHs, PAP-1 produced a low level of PO activity (4.7 U) with more than 30% of proPO being cleaved (Fig. 15A, lane 2). In the presence of SPHs, there was a 5-fold increase in PO activity (21 U) and a moderate increase of cleavage of proPO (Fig. 15A, lane 3). The negative control did not show any proPO cleavage (Fig. 15A, lane 1) or significant PO activity (1.7 U) (Fig. 15B). This result demonstrated that *M. sexta* SPHs also increased the generation of PO activity from *B. mori* proPO.

CHAPTER V

DISCUSSION

ProPO activation is one of the immune pathways by which arthropods defend themselves against microbial infections. This process has been extensively studied in the beetles, silkworm, and tobacco hornworm (Kwon et al., 2000; Lee et al., 1998; Satoh et al., 1999; Jiang et al., 1998; Jiang et al., 2003a; Jiang et al, 2003b). Mechanisms for proPO activation are not well understood and somewhat controversial. As described earlier in the literature review, active silkworm PO resulted from cleavage at the first site of proPO, whereas a secondary cleavage is required for yielding PO in the beetles. In the tobacco hornworm, the secondary cleavage in proPO-p1 is observed under certain conditions (data not shown), but its role in generating active PO is still uncertain. In this work, I have improved the scheme for isolating PAP-1 from *M. sexta* prepupal cuticles, purified it to near homogeneity, and characterized the proteinase biochemically. I have also attempted to understand the relationship between proteolysis and activation of proPO using the purified PAP-1, SPHs, and proPO.

I discovered that proPO cleavage does not directly correlate with its activation. PAP-1 itself cleaved more than 80% of proPO without generating significant amount of PO activity (Fig. 9). Previous studies showed that proPO can be activated by detergents or alcohols (Asada et al., 1993; Hall et al., 1995). But my results ruled out this possibility, as I have found that cleavage is critical for proPO activation by PAP-1 (Fig.

10). The same phenomenon was observed in the case of PAP-2 and PAP-3 (data not shown). Therefore, we suggest that proteolysis of proPO and its activation are two different processes: activation of proPO requires proteolysis but proteolysis can occur without producing active PO.

We still do not understand why cleaved proPO can be inactive and what the role of SPHs is in generating active PO. One possible model is that cleaved proPO may have different association states and the oligomers represent active PO. SPHs and active PAP-1 may bind to proPO and induce an active conformation while cleavage occurs. Such cleavage products form high molecular weight PO oligomers (Fig. 11). This model is consistent with our current data, indicating that PO activity and proPO cleavage are not parallel. In the absence of SPHs, active PAP-1 cleaves proPO but the product is perhaps in an inactive/transient conformation that cannot form oligomers.

This model further satisfies our results where SPHs cannot generate PO activity in the presence of inactivated PAP-1 (Fig. 12). Although there may be correct interactions between SPHs/PAP-1 and proPO, no cleavage could occur to produce active PO. PAP-1/SPHs may not interact with cleaved proPO, either.

The third experiment also satisfies our model, where both active PAP-1 and SPHs were present but due to insignificant amount of proPO not much PO activity was detected (Fig. 14). The formation of PO oligomers (Fig. 11) in the presence of SPHs in the case of PAP-1 further revealed the association states of active PO. Future studies may show the different association states of PO produced in the absence or presence of SPHs by using native gel electrophoretic analysis of the cleavage products.

At a high ionic strength, both PO activity and proPO activation were affected. The reduced PO activity at higher ionic strengths suggests that the association states of active PO might have been disrupted. The reduced proPO cleavage and activation suggests that the molecular interactions among proPO, PAP-1 and SPHs are impaired and electrostatic forces could play a role in these interactions.

It was shown (Fig. 14) that SPHs failed to generate active PO in the presence of cleaved proPO and active PAP-1. This suggests that SPHs act at proPO level and the N-terminal propeptide of proPO is involved in SPH binding. This might be the reason why SPHs did not bring change either in the K_M or V_{max} of PAP-1 towards IEARpNA but have shown enhanced effect towards proPO since IEAR*p*NA is a small peptide whose activation side is similar to the activation site of proPO. Here we also suggest that SPHs might interact with proPO but not cleaved proPO to generate PO activity. We would like to further investigate the binding sites of proPO and SPHs by employing yeast two hybrid or immunoprecipitation techniques.

Furthermore, SPHs isolated from *M. sexta* consists of two proteins which it has not been possible to separate. We would like to test with recombinant SPHs whether SPH-1/SPH-2 or both are important for interaction with proPO/PAP-1. It is reported that protease-like domain of SPHs interacts with IML-2, PAP-1 and proPO (Yu et al., 2003). However, the function of clip domain remains to be studied.

According to the works in *B. mori*, activation of proPO is catalyzed by PPAE and does not require any protein factor, while this is not the case in *H. diomphalia* and *M. sexta*. Here, I demonstrated that *M. sexta* SPHs have positive effect on *B. mori* proPO activation and there was a 5-fold increase in PO activity and moderate increase of

cleavage in the presence of SPHs (Fig. 15). This suggests a similar mechanism of *M. sexta* proPO activation might also exist in *B. mori*.

Localization of proPO activation near the invading site of microbial infection is an important aspect of insect defense mechanism. It may result in high PO activity and prevent the toxic quinones from killing the host cells. It is likely that proPO activation is a tightly regulated process where SPHs are the key players. If proPAP-1 (no microbial invasion) somehow gets activated to PAP-1, PAP-1 escapes the serpin inhibition and cleaves the proPO, it may still not be able to produce active PO due to the absence of SPHs, whereas in response to microbial invasion, SPHs are produced to interact with active PAP-1 and proPO (Yu et al., 2003) and generate active PO near the surface of the bacteria.

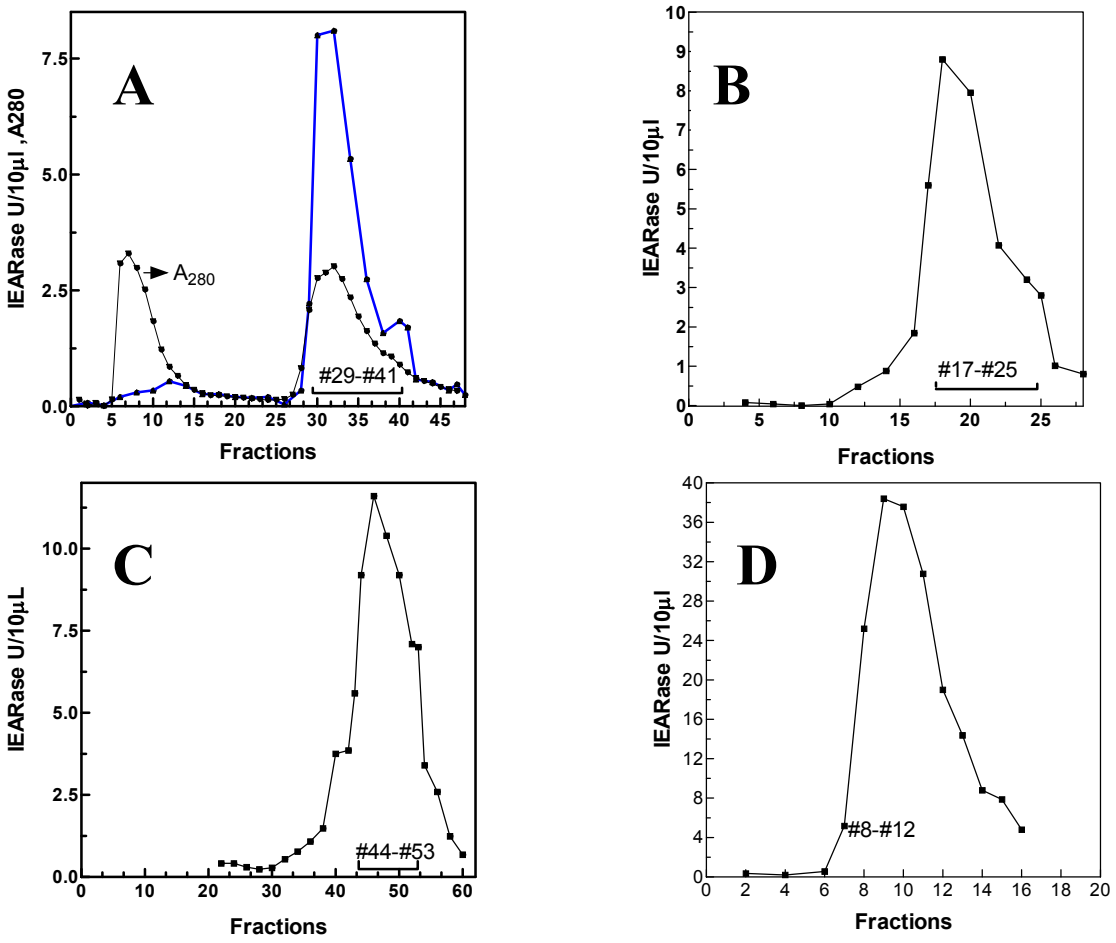


Fig. 1 Purification of PAP-1 from *M. sexta* prepupal cuticles

As described in Materials and Methods, 15-45% of ammonium sulfate-fraction of the cuticle extract was separated by hydroxyapatite (A), dextran sulfate (B), sephacryl S100-HR (C), and jacalin affinity (D) column chromatography. $A_{280\ nm}$ is represented by▲ ----▲; amidase activity is represented by■ -----■. The bar in the graphs represents the pooled fractions.

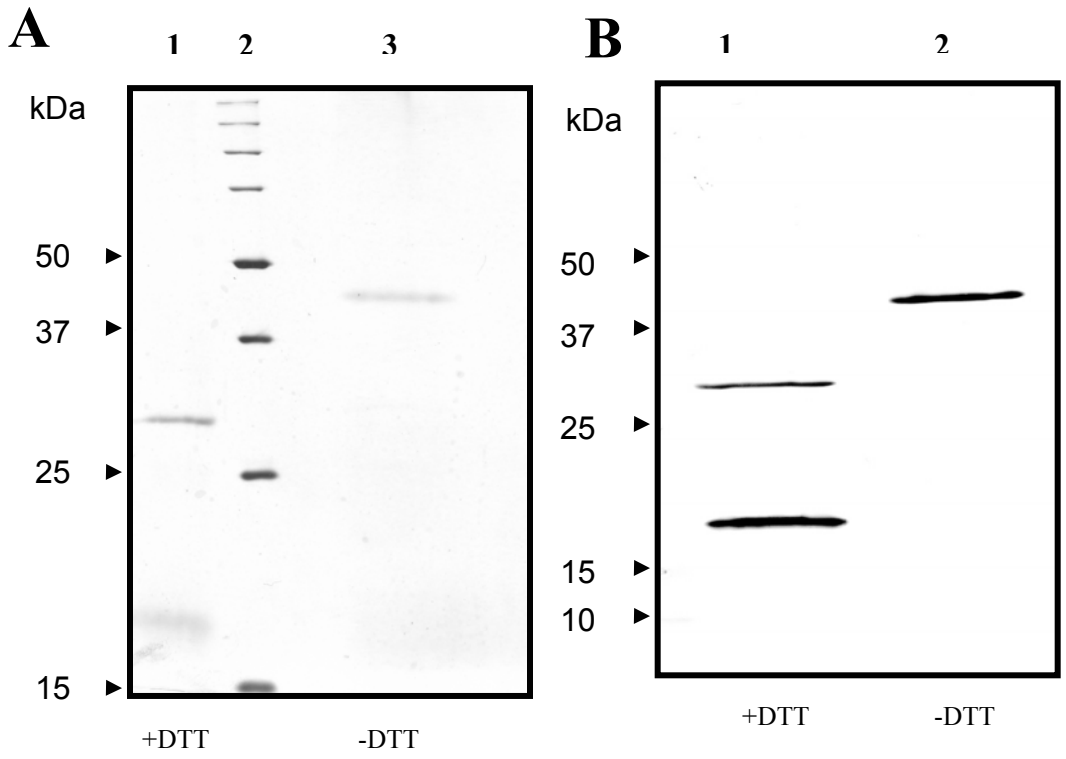


Fig.2 Characterization of purified PAP-1

SDS Polyacrylamide gel electrophoresis of PAP-1 purified from prepupal cuticles of *M. sexta*. Panel A) 12 µl of PAP-1 was loaded and separated by 12% gel and visualized by silver staining. Lane 1and 3, contain the PAP-1 under reducing and non reducing condition respectively. Lane 2, molecular weight standards B) Immunoblot analysis of PAP-1 was performed on 15 % SDS-PAGE gel by using 1: 2000 diluted antiserum against PAP-1 as the first antibody. Lane 1 and 2, PAP-1 under reducing and nonreducing condition, respectively. Positions and sizes of the molecular weight standards are marked on left side.

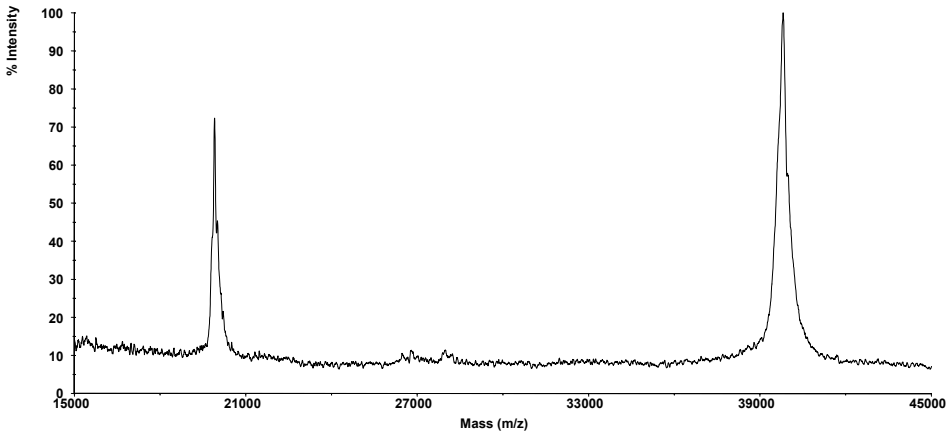


Fig. 3 MALDI-TOF mass spectrometry of *M. sexta* PAP-1

The procedures for sample preparation and mass spectrometry of PAP-1 purified were described in Materials and Methods. A representative strong single accumulation spectrum for PAP-1 is presented. Calibration of the spectrum was done with an external bovine serum albumin standard and subjected to noise removal. The first peak (left) represents the MH^{+2} and the second one represents the MH^{+}.

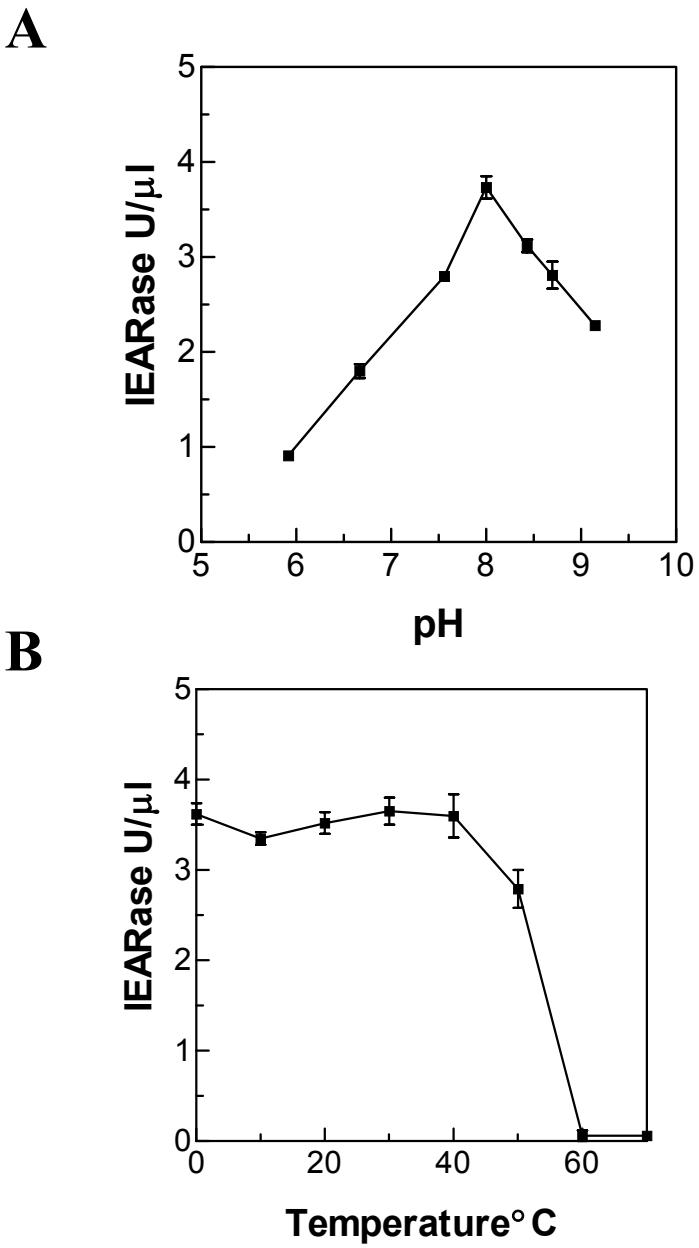


Fig. 4 pH and heat stability of PAP-1

A) Optimum pH of PAP-1: PAP-1(20 ng) was mixed with 1:5 diluted PBE96 adjusted to various pH's. Amidase activity was measured using IEAR*p*NA as a substrate.
B) Heat stability of PAP-1: PAP-1(20 ng) placed at different temperatures (0-70ºC) for 5 min, and amidase activity was measured using IEAR*p*NA. Each point represents mean ±S.D. (n=3).

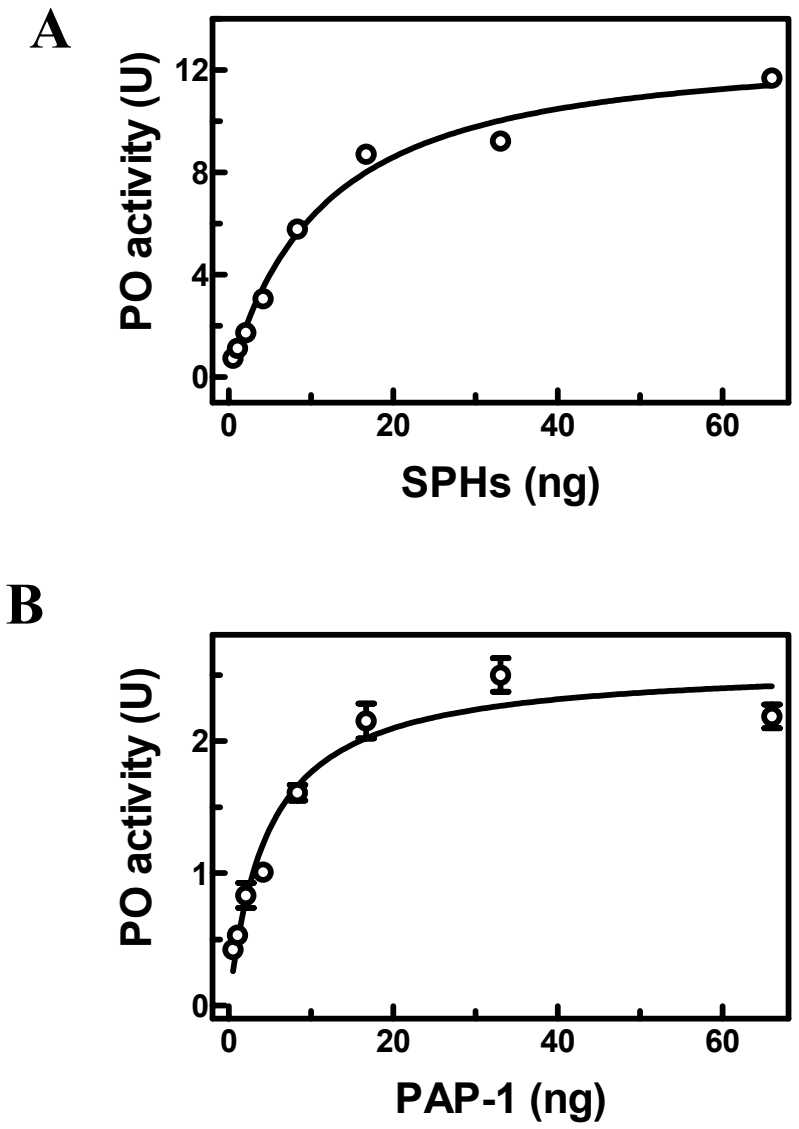
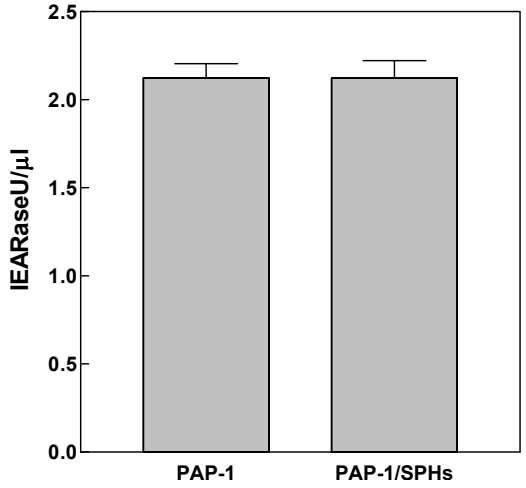


Fig. 5A) Effect of SPHs on proPO activation. To the fixed amount of PAP-1 (20 ng/µl) and proPO (0.1 µg/10µl), increased amount of SPHs were added. The reaction was placed on ice for 1h. 150 µl of dopamine was added and PO activities were measured. PO activities were plotted against the amount of SPHs. B) **Effect of PAP-1 on proPO activation**. Following the same procedure as described above except that PAP-1 was replaced with SPHs and vice-versa, PO activities were measured and plotted against the amount of PAP-1. Each point represents mean ±S.D. (n=3)

A



B

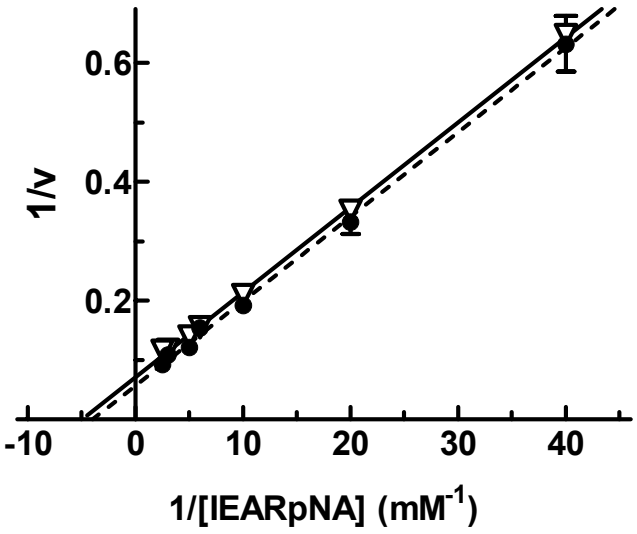


Fig. 6 Measurement of enzymatic properties of PAP-1

The experimental details were described in "Materials and methods". A) Effect of SPHs on the amidase activity of PAP-1. Determination of Michaelis Menten constant from double reciprocal plot towards B) IEAR*p*NA. In panel B) in the absence of SPHs, ●----●, in the presence of SPHs, Δ -------Δ; Data was analyzed by linear regression by using least square method. Each point represents mean ±S.D. (n=3)

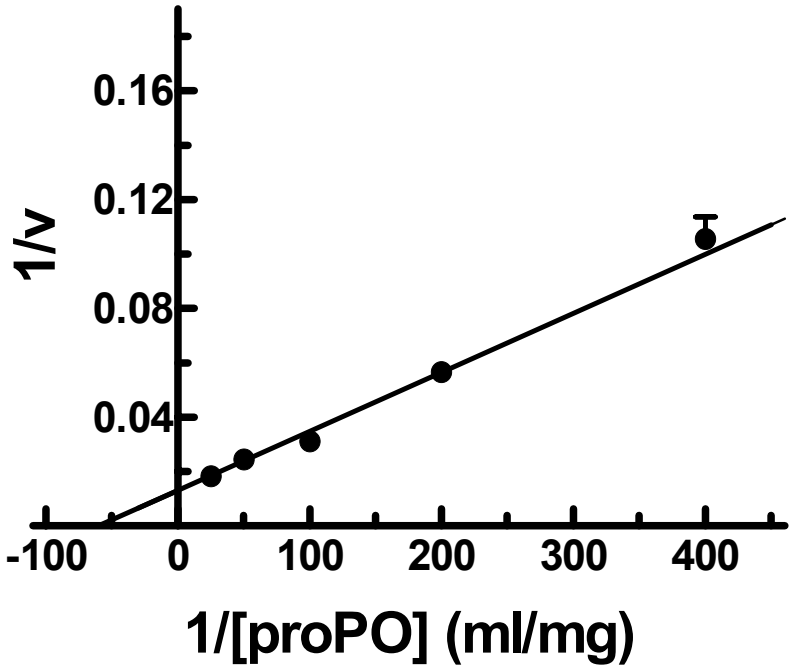


Fig 6C Measurement of enzymatic properties of PAP-1

Determination of Michaelis Menten constant from double reciprocal plot towards proPO. The experimental details are described in "Materials and methods". In panel A) in the presence of PAP-1 and SPHs, ●----●. Data was analyzed by linear regression by using least square method. Each point represents mean ±S.D. (n=2).

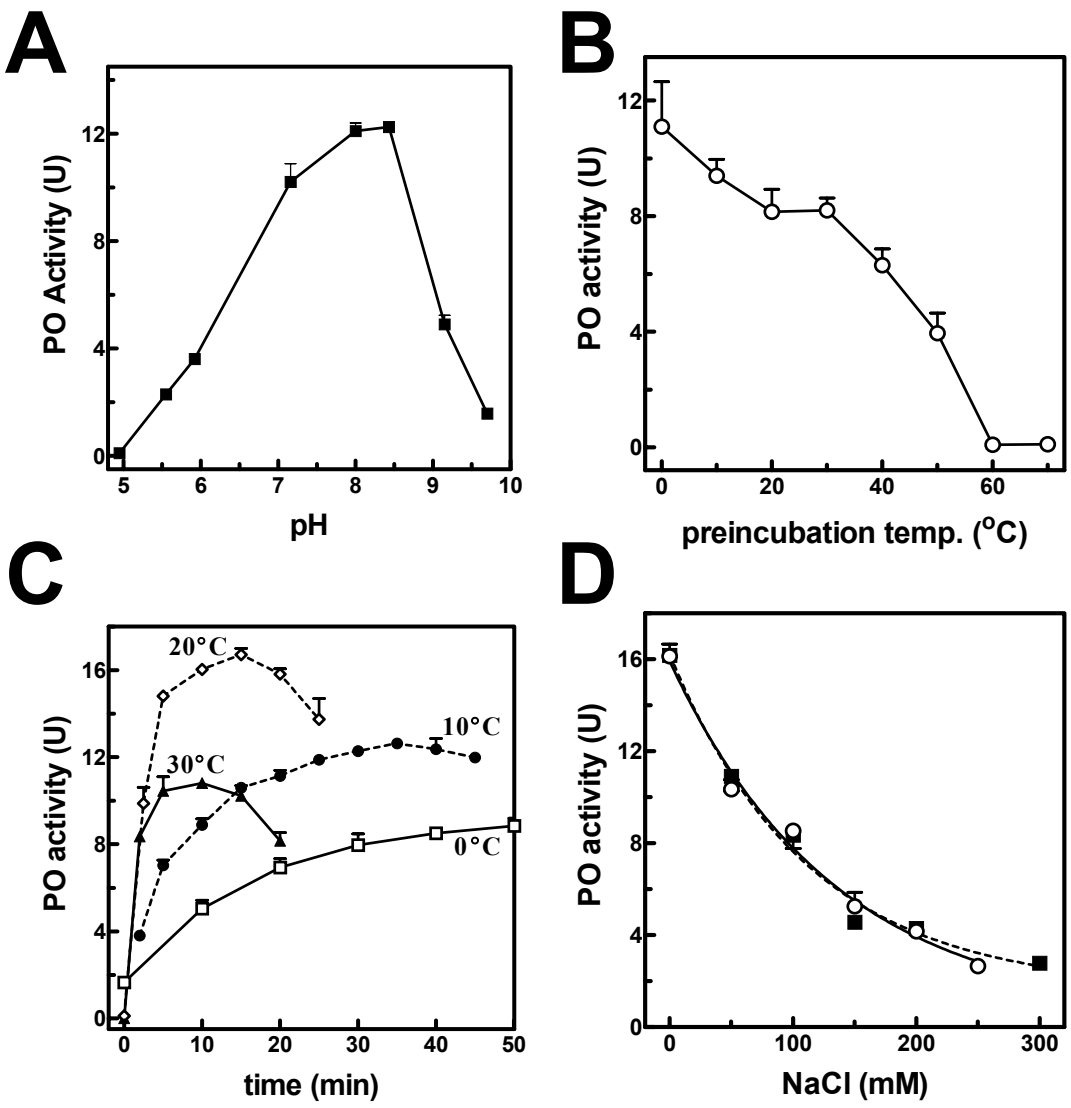


Fig. 7 Examination of optimal conditions for proPO activation:

As described in "Materials and methods", PAP-1 (20 ng/µl), SPHs (20 ng/µl), proPO (0.1 µg/µl) and buffer (20 mM Tris-Hcl, pH 8.0) mixed and placed on ice for 60 minutes. PO activity was measured and plotted on the Y-axis. On the X-axis, their respective parameters were plotted. Panel (A) pH optimum (B) heat stability of PAP-1 and SPHs (C) time course at 0, 10, 20 and 30 ºC (D) effects of salt concentration on proPO activation ■--------■ and PO activity ○ ------○. Each point represents mean ±S.D. (n=3)

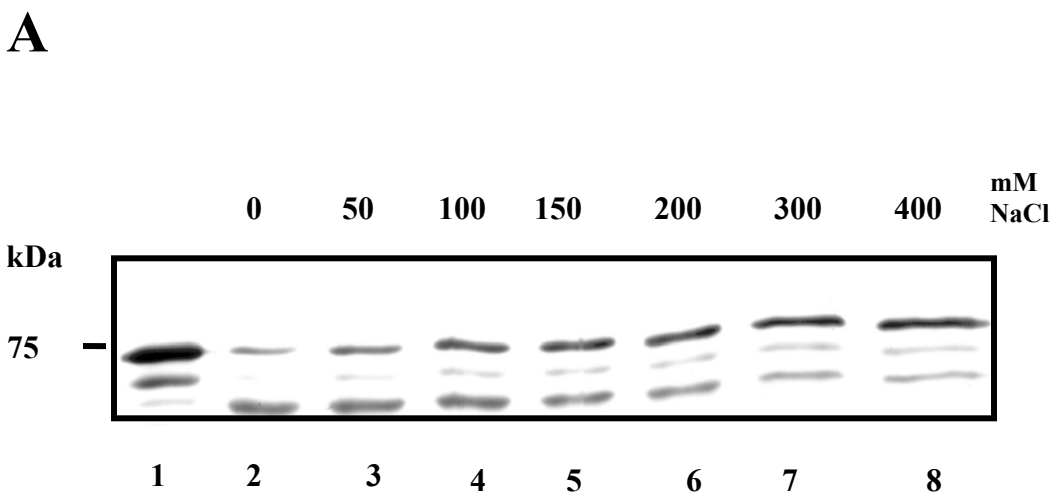


Fig. 8A Effect of ionic strength on the cleavage of proPO

100 ng of proPO was incubated with mixtures of 20 ng of PAP-1 and SPHs at 0 (*lane 2*), 50 (*lane 3*), 100 (*lane 4*), 150 (*lane 5*), 200 (*lane 6*), 300 mM (*lane 7*) and 400 mM (*lane 8*) of NaCl in 20 mM Tris-Hcl, pH 8.0 on ice for 1 h. The control proPO only (*lane 1*) was incubated in 20mM Tris pH 8.0 on ice for 1 h. The reaction mixture was analyzed by 6% SDS-PAGE gel, followed by immunoblot using 1: 2000 diluted antiserum against proPOas the first antibody.

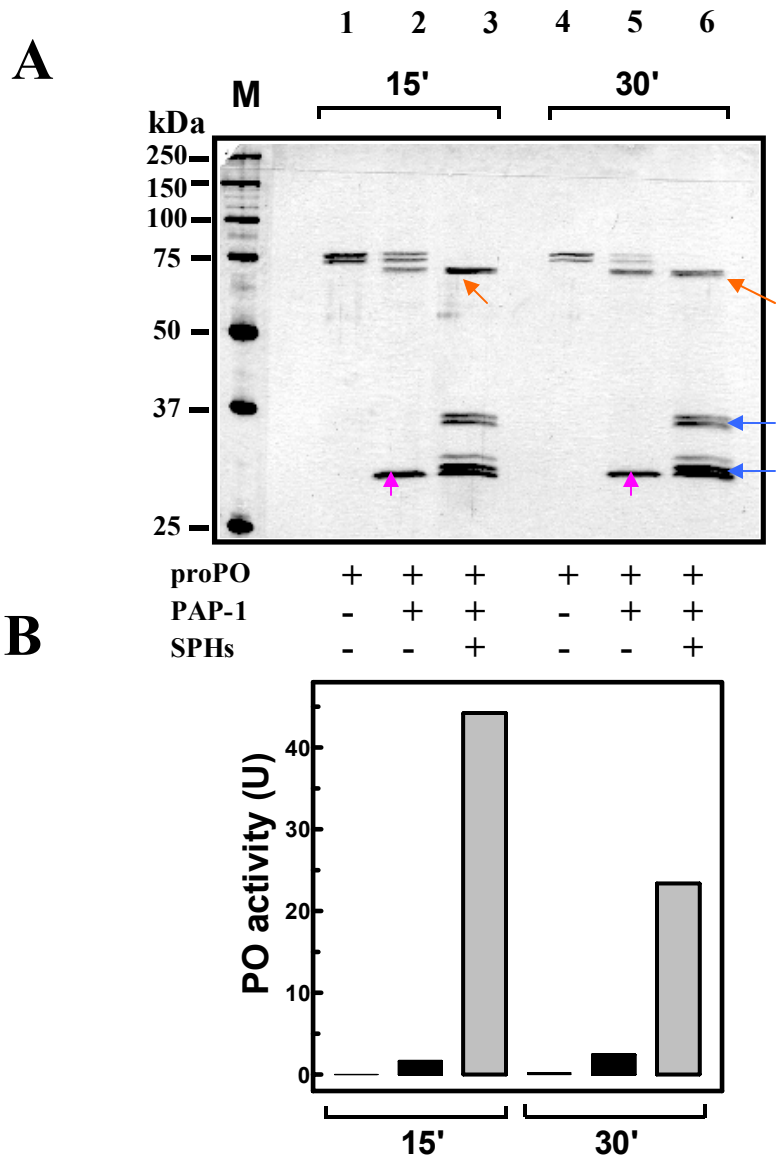


Fig. 9 Inspection of the relationship between proPO cleavage and PO activity:

A) immunoblot analysis. B) PO activity assay. 100 ng of purified proPO was incubated with buffer (*lanes 1 and 4*) or with 40 ng of PAP-1(*lanes 2 and 5*), or with mixtures of PAP-1 and SPHs containing 40 ng each (*lanes 3 and 6*) in 20 mM Tris-Hcl, pH 8.0 at room temperature for 15 and 30 min. PO activity was determined by using dopamine as a substrate. For immunoblot analysis, the activation mixtures contain 0.001% PTU and separated by 10% gel electrophoresis under the reducing condition. Immunoblot analysis was performed using 1: 2000 diluted antiserum against proPO as the first antibody. Protein molecular weight standards were marked on the left side. Red arrow indicates the cleaved proPO, pink indicates PAP-1 and blue indicates SPHs.

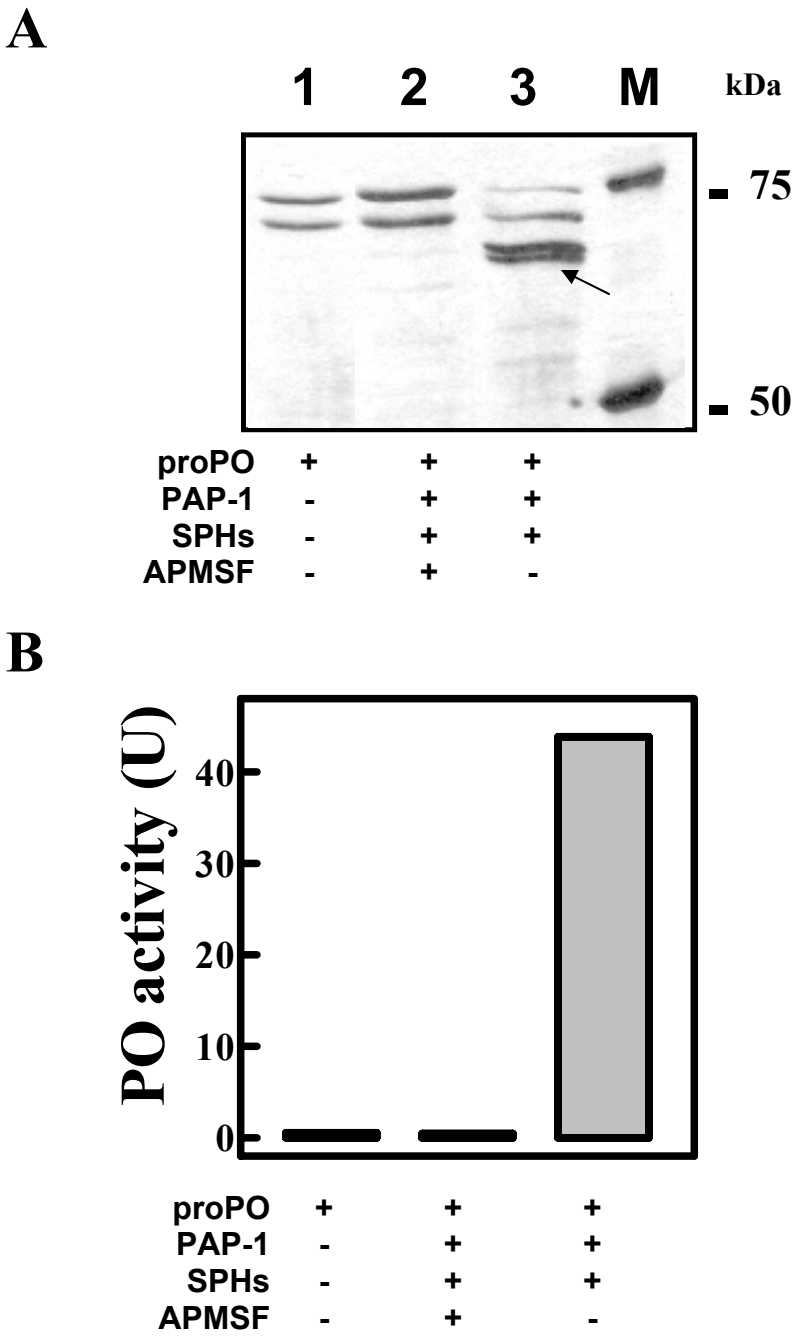


Fig. 10 Examination of the significance of limited proteolysis in proPO activation

(A) SDS-PAGE analysis (B) PO activity assay
As described in "Materials and methods", PAP-1(40 ng, 2μl) was inactivated by APMSF (8 mM, 2 μl) at 0ºC for 10 min and then SPHs (40 ng, 2μl) and proPO (0.1 μg, 10μl) were added and waited for 50 min. Samples were separated by 6% SDS-PAGE and visualized by silver staining. proPO only *(lane 1)*; proPO+ PAP-1 + APMSF +SPHs *(lane 2);* proPO +PAP-1+SPHs *(lane 3)* and protein markers *(lane M).* Arrow indicates the cleaved proPO.

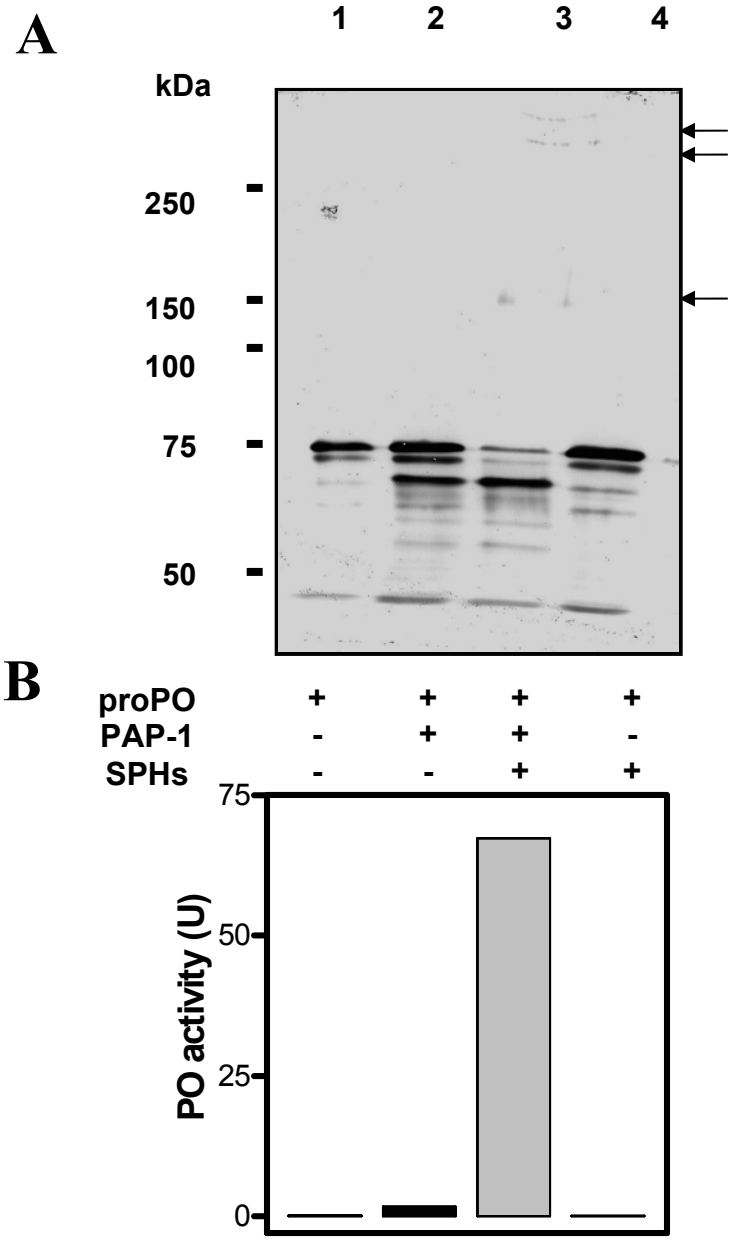


Fig. 11 Formation of PO oligomers in the presence of SPHs

A) immunoblot analysis. B) PO activity assay. 100 ng of purified proPO was incubated with buffer (*lane 1*), 40 ng of PAP-1(*lane 2*), mixtures of PAP-1 (40 ng) and SPHs (40 ng) (*lane 3*), or with 40 ng of SPHs in 20 mM Tris-Hcl, pH 8.0. PO activity was determined after incubating the mixtures on ice for 1 h by using dopamine. The activation mixtures were subjected to SDS-polyacrylamide gel electrophoresis under reducing condition. Immunoblot analysis was performed using 1: 2000 diluted antiserum against proPO as the first antibody. Sizes and positions of the protein molecular weight standards were indicated on the left side.

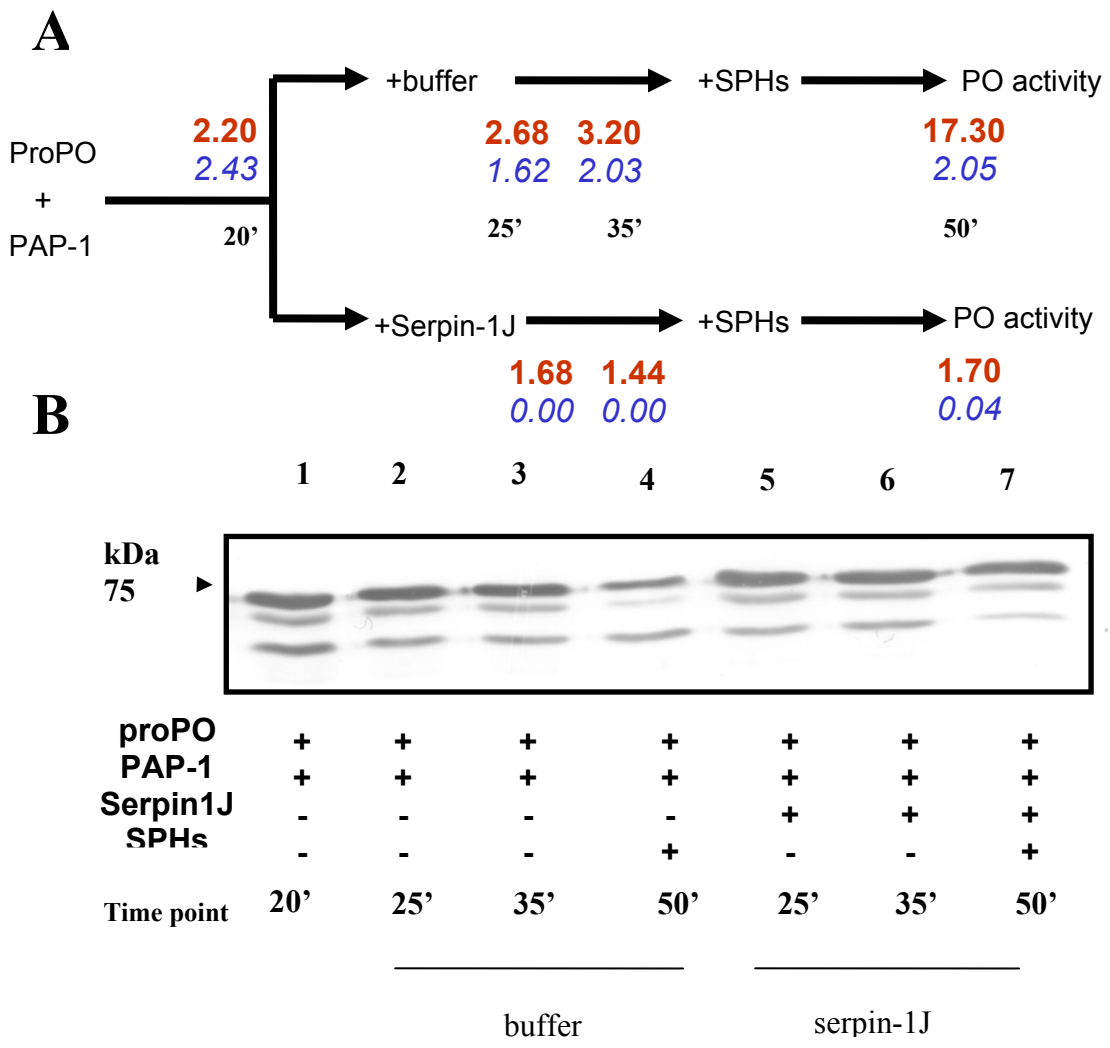


Fig 12 Investigation of a possible interaction between cleaved proPO and SPHs in the presence of inactivated PAP-1 by serpin-1J.

A) proPO activation scheme and enzyme activities B) immunoblot analysis
PAP-1(20 g) and proPO (100 ng) incubated on ice for 20 min and then serpin-1J (3 µg) was added. After 15 min, SPHs (20 ng) were added and incubated for another 15 min. At each time point, PO activity (**bold**) and amidase activity (*italicized*) and were determined. For immunoblot analysis, samples were separated by 6% gel. proPO + PAP-1 at 20 min *(lane 6)*, proPO + PAP-1 + serpin 1J at 25 min *(lane 6)*, proPO + PAP-1 + serpin 1J at 35 min *(lane 7)*, proPO + PAP-1 + serpin 1J + SPHs min at 50 min *(lane 8)*. The controls (buffer) followed the same order and loaded on *(lanes 2, 3, 4)*. Immunoblot was performed by using 1: 2000 diluted antiserum against proPO as the first antibody.

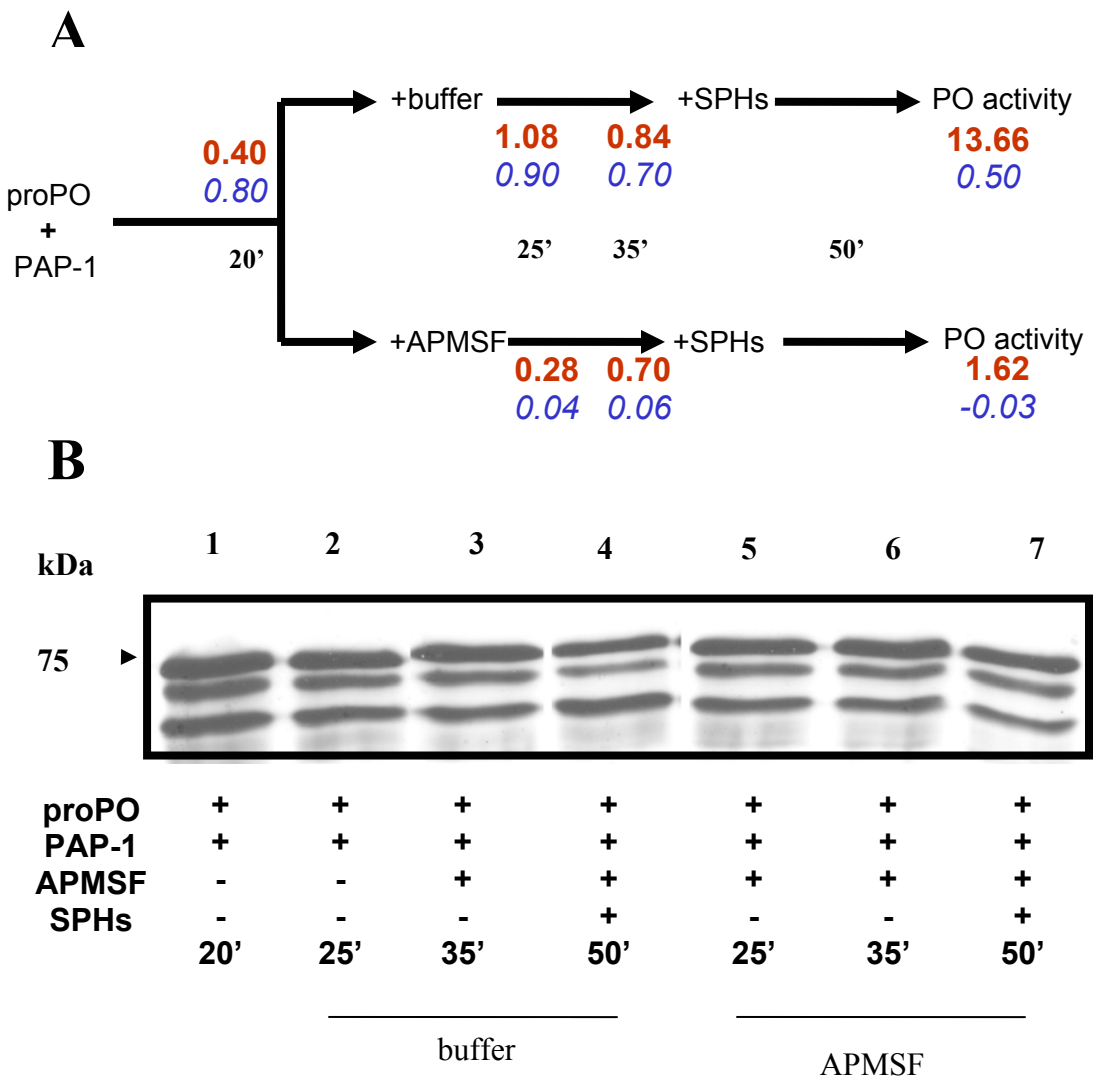


Fig 13 Investigation of a possible interaction between cleaved proPO and SPHs in the presence of inactivated PAP-1 by APMSF

A) proPO activation scheme and enzyme activities B) immunoblot analysis
PAP-1(20 g) and proPO (100 ng) incubated on ice for 20 min and then APMSF (8 mM) was added. After 15 min, SPHs (20 ng) were added and incubated for another 15 min. At each time point, PO activity (**bold**) and amidase activity (*italicized*) and were determined. For immunoblot analysis, samples were separated by 6% gel. proPO + PAP-1 at 20 min *(lane 6)*, proPO + PAP-1 + APMSF at 25 min *(lane 6)*, proPO + PAP-1 + APMSF at 35 min *(lane 7)*, proPO + PAP-1 + APMSF + SPHs min at 50 min *(lane 8)*. The controls (buffer) followed the same order and loaded on *(lanes 2, 3, 4)*. Immunoblot was performed by using 1: 2000 diluted antiserum against proPO as the first antibody.

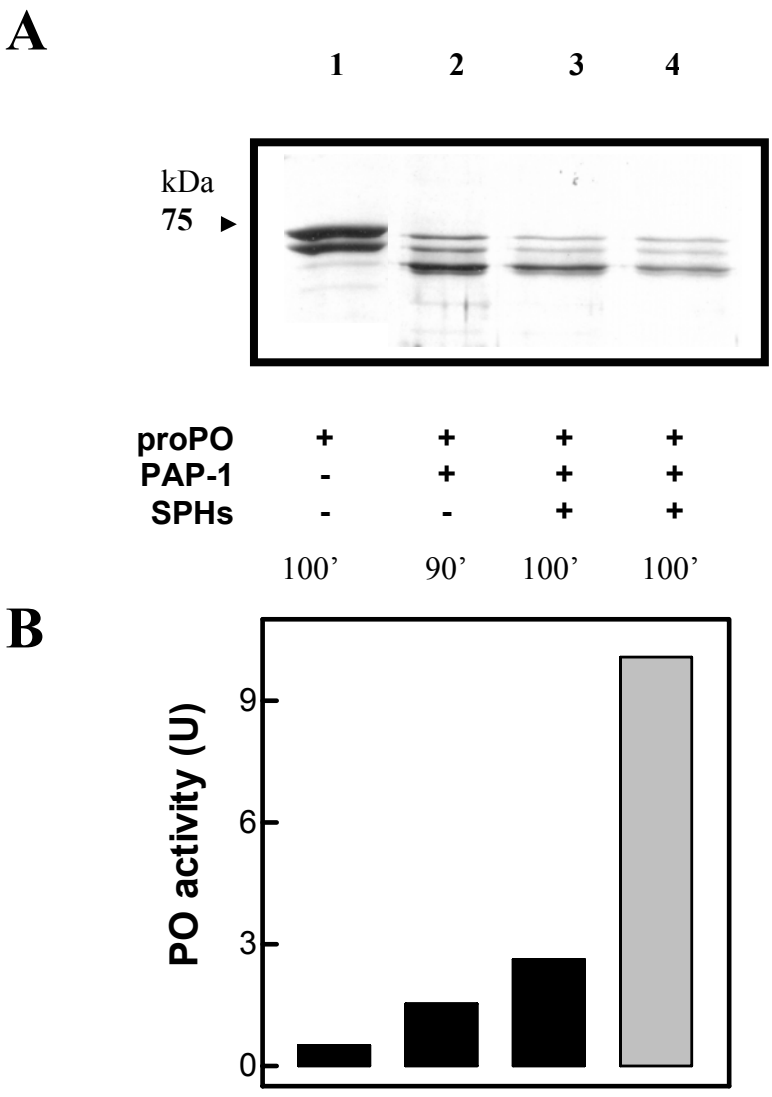


Fig.14 **Possible interaction of SPHs with cleaved proPO in the presence of active PAP-1**

A) SDS-PAGE analysis B) PO activity assay.
PAP-1 (40 ng) and proPO (100 ng) combined and incubated for 90 min *(lane 2)* on ice. Then SPHs was added (40 ng) and incubated for another 10 min *(lane 3)*. In the control proPO, PAP-1 and SPHs were mixed at the same time and incubated for 100 min *(lane 4)*. PO activity was measured using dopamine as a substrate. For SDS PAGE analysis, the samples were mixed with sample buffer, separated by electrophoresis and visualized by silver staining.

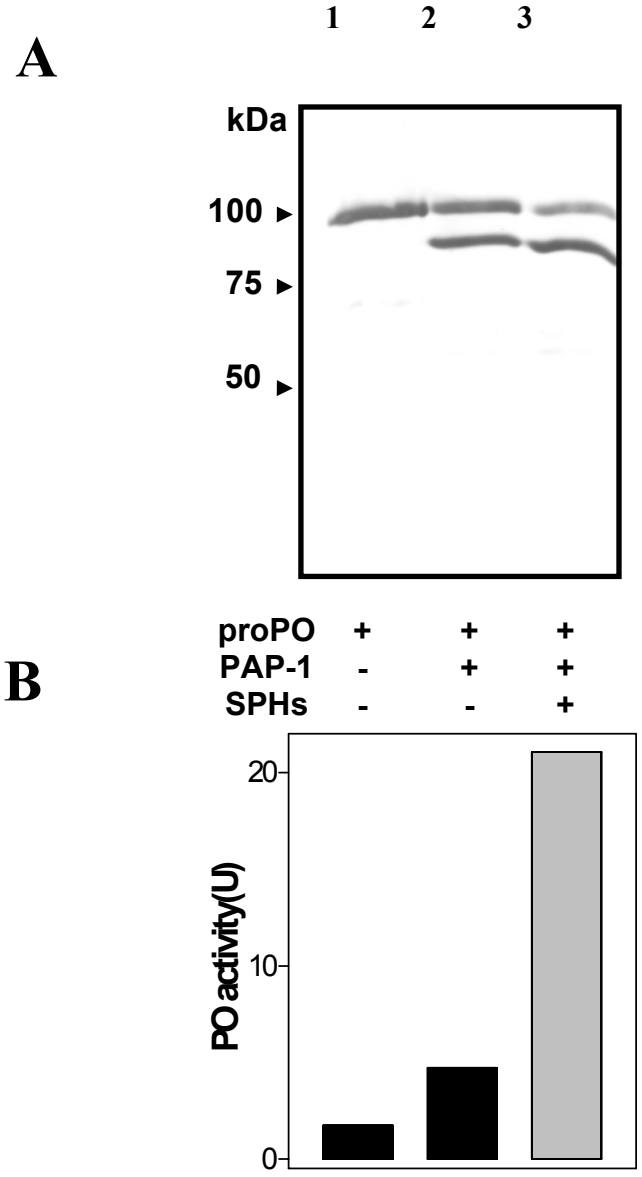


Fig. 15 **Effect of *M.sexta* SPHs on silkworm proPO activation**

A) immunoblot analysis B) PO activity assay.
200 ng of silkworm proPO was incubated with buffer (*lane 1*), PAP-1 (40 ng)(*lane 2*), mixtures of PAP-1 (40 ng) and SPHs (40 ng) (*lane 3*) in 20 mM Tris-Hcl, pH 8.0, 5 mM $CaCl_2$. PO activity was determined after incubating the mixtures at room temperature for 15 min. For immunoblot analysis, the samples were mixed with PTU (0.001%) and separated on 7.5% gel. The membrane was treated with 1:2000 diluted antiserum against *M.sexta* proPO as the first antibody. Sizes and positions of the protein molecular weight standards were indicated on the left side.

CHAPTER VI

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